

56/T3/00  
JCE99 U.S. PTO

Practitioner's Docket No. 46906-2 DIV

**PATENT**

A  
JC612 U.S. PTO  
09/282879  
03/31/99

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 20231

**NEW APPLICATION TRANSMITTAL**

Transmitted herewith for filing is the patent application of

Inventor(s): **Subroto CHATTERJEE**

**WARNING:** 37 C.F.R. § 1.41(a)(1) points out:

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(l) is filed supplying or changing the name or names of the inventor or inventors."

For (title): **RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME**

---

**CERTIFICATION UNDER 37 C.F.R. § 1.10\***

(Express Mail label number is **mandatory**.)

(Express Mail certification is **optional**.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date March 31, 1999, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number E1978002454US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Landry

(type or print name of person mailing paper)

  
Signature of person mailing paper

**WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

**\*WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

## 1. Type of Application

This new application is for a(n)

(check one applicable item below)

- Original (nonprovisional)
- Design
- Plant

**WARNING:** *Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

**WARNING:** *Do not use this transmittal for the filing of a provisional application.*

**NOTE:** *If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.*

- Divisional.
- Continuation.
- Continuation-in-part (C-I-P).

## 2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

**NOTE:** *A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:*

*(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or*

*(ii) Complete as set forth in § 1.51(b); or*

*(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or*

*(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).*

*37 C.F.R. § 1.78(a)(1).*

**NOTE:** *If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.*

**WARNING:** *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

**WARNING:** When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application must be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).

The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

### 3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

37 Pages of specification

4 Pages of claims

7 Sheets of drawing

**WARNING:** DO NOT submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).

**NOTE:** "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

formal

informal

### B. Other Papers Enclosed

3 Pages of declaration and power of attorney

1 Pages of abstract

  Other

### 4. Additional papers enclosed

Amendment to claims

Cancel in this applications claims 1-12 before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

Preliminary Amendment

Information Disclosure Statement (37 C.F.R. § 1.98)

Form PTO-1449 (PTO/SB/08A and 08B)

Citations

- Declaration of Biological Deposit
- Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
- Authorization of Attorney(s) to Accept and Follow Instructions from Representative
- Special Comments
- Other

#### 5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)–(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)–(4).

Enclosed Copy filed in the parent nonprovisional application

Executed by

(check all applicable boxes)

- inventor(s).
- legal representative of inventor(s).  
37 C.F.R. §§ 1.42 or 1.43.
- joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.
  - This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.
- Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

- Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of all the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

- Showing that the filing is authorized.  
(not required unless called into question, 37 C.F.R. § 1.41(d))

## 6. Inventorship Statement

**WARNING:** If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.

The inventorship for all the claims in this application are:

The same.

or

Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,  
 is submitted.  
 will be submitted.

## 7. Language

**NOTE:** An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

English

Non-English

The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

## 8. Assignment

An assignment of the invention to Johns Hopkins University of Baltimore  
Maryland (copy filed with the parent nonprovisional application enclosed)

is attached. A separate  "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or  FORM PTO 1595 is also attached. (copy)

will follow.

**NOTE:** "If an assignment is submitted with a new application, send two separate letters—one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

**WARNING:** A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.

(New Application Transmittal [4-1]—page 5 of 11)

## 9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Country	Appln. No.	Filed
Country	Appln. No.	Filed

from which priority is claimed

- is (are) attached.
- will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

## 10. Fee Calculation (37 C.F.R. § 1.16)

### A. Regular application

CLAIMS AS FILED					
Number filed	Number Extra	Rate	Basic Fee	37 C.F.R. 1.16(a)	
				\$760.00	
<b>Total</b>					
Claims (37 C.F.R. § 1.16(c))	18 - 20 = 0	×	\$ 18.00		
<b>Independent</b>					
Claims (37 C.F.R. § 1.16(b))	4 - 3 = 1	×	\$ 78.00	78.00	
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))		+	\$260.00		

- Amendment cancelling extra claims is enclosed.
- Amendment deleting multiple-dependencies is enclosed.
- Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation \$ 838.00

B.  Design application  
(\$310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation \$ \_\_\_\_\_

C.  Plant application  
(\$480.00—37 C.F.R. § 1.16(g))

Filing fee calculation \$ \_\_\_\_\_

**11. Small Entity Statement(s)**

Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

**WARNING:** "Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).

**WARNING:** "Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).

(complete the following, if applicable)

Status as a small entity was claimed in prior application

08/774,104, filed on 12/24/96, from which benefit is being claimed for this application under:

35 U.S.C. §  119(e),  
 120,  
 121,  
 365(c),

and which status as a small entity is still proper and desired.

A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of A, B or C above)

\$ 419.00

**NOTE:** Any excess of the full fee paid will be refunded if small entity status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

**12. Request for International-Type Search (37 C.F.R. § 1.104(d))**

(complete, if applicable)

Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

**13. Fee Payment Being Made at This Time**

Not Enclosed

No filing fee is to be paid at this time.

*(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)*

Enclosed

Filing fee

\$ 419.00

Recording assignment

*(\$40.00; 37 C.F.R. § 1.21(h))*

*(See attached "COVER SHEET FOR  
ASSIGNMENT ACCOMPANYING NEW  
APPLICATION".)*

\$ \_\_\_\_\_

Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached

*(\$130.00; 37 C.F.R. §§ 1.47 and 1.17(i))*

\$ \_\_\_\_\_

For processing an application with a specification in a non-English language

*(\$130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))*

\$ \_\_\_\_\_

Processing and retention fee

*(\$130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))*

\$ \_\_\_\_\_

Fee for international-type search report

*(\$40.00; 37 C.F.R. § 1.21(e))*

\$ \_\_\_\_\_

*NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).*

Total fees enclosed

\$ 419.00

**14. Method of Payment of Fees**

Check in the amount of \$ 419.00

Charge Account No. \_\_\_\_\_ in the amount of  
\$ \_\_\_\_\_

A duplicate of this transmittal is attached.

*NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).*

## 15. Authorization to Charge Additional Fees

**WARNING:** If no fees are to be paid on filing, the following items should not be completed.

**WARNING:** Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 04-1105:

37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

**NOTE:** Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).

37 C.F.R. § 1.17 (application processing fees)

**NOTE:** ". . . A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

**NOTE:** Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

**NOTE:** 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . ." From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

**16. Instructions as to Overpayment**

**NOTE:** ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

Credit Account No. 04-1105  
 Refund



**SIGNATURE OF PRACTITIONER**

Reg. No. 33,860

**Peter F. Corless**

*(type or print name of attorney)*

Dike, Bronstein, Roberts & Cushman, LLP  
130 Water Street

P.O. Address

Customer No.

Boston, MA 02109

**Incorporation by reference of added pages**

*(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)*

Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added 23

Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added \_\_\_\_\_

Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added \_\_\_\_\_

**Statement Where No Further Pages Added**

*(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)*

This transmittal ends with this page.

**ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF  
PRIOR U.S. APPLICATION(S) CLAIMED**

NOTE: See 37 CFR 1.78.

**17. Relate Back**

**WARNING:** *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

Amend the specification by inserting, before the first line, the following sentence:

**A. 35 U.S.C. 119(e)**

**NOTE:** *"Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).*

"This application claims the benefit of U.S. Provisional Application(s) No(s).:

**APPLICATION NO(S):****FILING DATE**

\_\_\_\_ / \_\_\_\_\_  
\_\_\_\_ / \_\_\_\_\_  
\_\_\_\_ / \_\_\_\_\_

\_\_\_\_ "  
\_\_\_\_ "  
\_\_\_\_ "

**B. 35 U.S.C. 120, 121 and 365(c)**

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R § 1.78(a)(2).

"This application is a  
 continuation  
 continuation-in-part  
 divisional

of copending application(s)

application number 08/ 774,104 filed on 12/24/96"  
 International Application \_\_\_\_\_ filed on \_\_\_\_\_ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

"The nonprovisional application designated above, namely application \_\_\_\_\_ / \_\_\_\_\_, filed \_\_\_\_\_, claims the benefit of U.S. Provisional Application(s) No(s).: \_\_\_\_\_

**APPLICATION NO(S).:**

**FILING DATE**

\_\_\_\_\_/\_\_\_\_\_"  
\_\_\_\_\_/\_\_\_\_\_"  
\_\_\_\_\_/\_\_\_\_\_"

Where more than one reference is made above, please combine all references into one sentence.

## 18. Relate Back—35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appn. no.	Filed on
The certified copy(ies) has (have)		

- been filed on \_\_\_\_\_, in prior application O / \_\_\_\_\_, which was filed on \_\_\_\_\_.
- is (are) attached.

**WARNING:** *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

## 19. Maintenance of Copendency of Prior Application

**NOTE:** *The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).*

### A. Extension of time in prior application

*(This item must be completed and the papers filed in the prior application, if the period set in the prior application has run.)*

- A petition, fee and response extends the term in the pending prior application until \_\_\_\_\_.
- A copy of the petition filed in prior application is attached.

### B. Conditional Petition for Extension of Time in Prior Application

*(complete this item, if previous item not applicable)*

- A conditional petition for extension of time is being filed in the pending prior application.
- A copy of the conditional petition filed in the prior application is attached.

**20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed**

*(complete applicable item (a), (b) and/or (c) below)*

(a)  This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

the same.

less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

---

*(type name(s) of inventor(s) to be deleted)*

(b)  This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

the same.

the following additional inventor(s) have been added:

---

*(type name(s) of inventor(s) to be added)*

(c) The inventorship for all the claims in this application are

the same.

not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

is submitted.

will be submitted.

**21. Abandonment of Prior Application (if applicable)**

Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

**22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment**

**WARNING:** "The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b), 6th ed., rev.2.

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

**23. Small Entity (37 CFR § 1.28(a))**

Applicant has established small entity status by the filing of a statement in parent application 08 / 774,104 on 12/24/96.  
 A copy of the statement previously filed is included.

**WARNING:** See 37 CFR § 1.28(a).

**24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING**

A notification of the filing of this  
(check one of the following)

continuation  
 continuation-in-part  
 divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

(Added Pages for Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed  
[4-1.1]—page 5 of 5)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**In re application of: **S. Chatterjee**Serial No.: **0 8 / 774,104** Group No.: **1652**  
Filed: **December 24, 1996** Examiner: **L. Hobbs**For: **RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME****Assistant Commissioner for Patents**  
**Washington, D.C. 20231****NOTIFICATION OF FILING OF CONTINUING,  
DIVISIONAL OR CONTINUED PROSECUTION APPLICATION**

Notification is hereby being made of the filing of a:

continuation  
 continuation-in-part  
 divisional  
 continued prosecution

application for this case

concurrently herewith.  
 on \_\_\_\_\_

Date

**CERTIFICATION UNDER 37 CFR 1.8(a) and 1.10***(When using Express Mail, the Express Mail label number is mandatory;  
Express Mail certification is optional.)*

I hereby certify that, on the date shown below, this correspondence is being:

**MAILING**

deposited with the United States Postal Service in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

37 CFR 1.8(a)

37 CFR 1.10\*

with sufficient postage as first class mail.

as "Express Mail Post Office to Addressee"

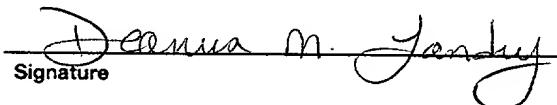
Mailing Label No. \_\_\_\_\_ (mandatory)

ET1978002454US

**TRANSMISSION**

transmitted by facsimile to the Patent and Trademark Office.

Signature

  
**Deanna M. Landry**

(type or print name of person certifying)

**\*WARNING:** Each paper or fee filed by Express Mail **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

(Notification of Filing of Continuing, Divisional or Continued Prosecution Application [4-9] (page 1 of 2))



**SIGNATURE OF PRACTITIONER**

Reg. No. **33,860**

**Peter F. Corless**

*(type or print name of practitioner)*

Tel. No.: (617) 523-3400

**Dike, Bronstein, Roberts & Cushman, LLP**

**130 Water Street**

P.O. Address

Customer No.:

**Boston, MA 02109**

04222329-032496

Applicant or Patentee: Subroto B. Chatterjee

Attorney Docket No.

Serial or Patent No.:

Filed or Issued:

For: Recombinant N-Smase and Nucleic Acids Encoding Same

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27(D)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE

Address of Organization: 720 Rutland Avenue

Baltimore, Maryland 21205

Type of Organization:

UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION  
 TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))  
 NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA  
(NAME OF STATE: )  
(CITATION OF STATUTE: )  
 WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA  
 WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
(NAME OF STATE: )  
(CITATION OF STATUTE: )

Thereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Recombinant N-Smase and Nucleic Acids Encoding Same by inventor(s) Subroto B. Chatterjee described in

the specification filed herewith.  
 application serial no. \_\_\_\_\_, filed \_\_\_\_\_.  
 patent no. \_\_\_\_\_, issued \_\_\_\_\_.

Thereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: \_\_\_\_\_

Address: \_\_\_\_\_

INDIVIDUAL  SMALL BUSINESS CONCERN

NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Howard W. Califano, Esq.

Title: Assistant Dean and Director

Address: 2024 East Monument Street, Suite 2-100 Baltimore, Maryland 21205

Signature: Howard W. Califano Date: 12-20-96

Docket No. 46906-2  
Express Mail Label No.: EI978002454US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:  
Subroto Chatterjee

Serial No.: Not yet assigned

Filed: Herewith

RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

## PRELIMINARY AMENDMENT

Please amend the above-identified application filed herewith as follows.

## IN THE CLAIMS

Please cancel claims 1-12 without prejudice.

## REMARKS

Claims 1-12 have been cancelled without prejudice.

Early consideration and allowance of the application are earnestly solicited.

Respectfully submitted,

Peter F. Corless (Reg. No. 33,860)  
Attorney for Applicant  
Dike, Bronstein, Roberts & Cushman, LLP  
130 Water Street  
Boston, MA 02109  
(617) 523-3400

RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME

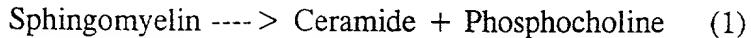
BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to human neutral sphingomyelinases (N-SMases), including recombinant N-SMases and fragments and derivatives thereof and isolated nucleic acids encoding N-SMases and fragments and derivatives. In preferred aspects, assays for identifying compounds that can modulate N-SMase related activity are provided, particularly assays to identify a pharmacological agent useful in the diagnosis or treatment of disorders associated with human neutral sphingomyelinases.

2. Background

10 Sphingomyelinases type-C (E.C. 3.1.4.12) are a group of phospholipases that catalyze the hydrolytic cleavage of sphingomyelin via the following reaction (1).



15 Native N-SMase purified from human urine and cultured human kidney proximal tubular cell membranes has an apparent molecular weight of 92 kDa, neutral pH optima, is heat unstable and is localized on the surface of various cells. S. Chatterjee, *Adv. Lipid Res.*, 26:25-48 (1993); S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989); and S. Chatterjee et al., *Methods in Enzymology*, 20 Phospholipase, 197:540-547 (1991). N-SMase action has been shown to mediate signal transduction of vitamin D<sub>3</sub>, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon-gamma and nerve growth factor (Y. Hannun, *J. Biol. Chem.*, 269:3,125-3,128 (1994); S. Chatterjee, *J. Biol. Chem.*, 268:3,401-3,406 (1993); and S. Chatterjee, *J. Biol. Chem.*, 269:879-882 (1994)) leading to cell differentiation in human leukemic (HL-60)

cells and insulin signaling (P. Peraldi et al., *J. Biol. Chem.*, 271:13018-13022 (1996)).

In addition to the biological roles of sphingomyelin and ceramide in signal transduction pathways involving cell regulation, recent evidence suggests that sphingomyelinases may be involved in the mobilization of cell surface cholesterol, in cholesterol ester synthesis, and in induction of low density lipoprotein (LDL) receptor activity. See S. Chatterjee, *Advances in Lipid Research*, 26:25-48 (1993). Recent evidence also supports a possible role of ceramide (a product of N-SMase activity) in programmed cell death and/or "apoptosis" and activation of the gene for nuclear factor (NF)-kB. See A. Alessenko and S. Chatterjee, *Mol. Cell. Biochem.*, 143:169-174 (1995). Sphingomyelinases are also believed to serve as a signal for various exogenous effectors such as antibiotics, drugs, and growth factors, which influence the normal physiology of cells.

15

A number of specific disorders have been associated with N-SMase. For example, N-SMase has been reported to be associated with insulin resistant diabetes and obesity. See Speigel et al., *J. Biol. Chem.*, 1996. N-SMase is also associated with malaria. The development of the malaria parasite plasmodium requires N-SMase. See Lauer et al., *Proc. Nat. Acad. Sci. (USA)*, 1995. N-SMase also is involved in liver cell proliferation. See Alessenko, Chatterjee, *Mol. Cell Biochem.*, 143:169-174 (1995).

Thus, methods for identifying agents which can modulate N-SMase activity would be highly useful. Moreover, methods for identifying pharmacological agents of interest by automated, high throughput drug screening have become increasing relied upon in a variety of pharmaceutical and biotechnology drug development programs. Unfortunately, however, requisite reagents for such high throughput screening assays to identify agents potentially useful in treatment of N-SMase associated disorders are not readily available. For example, current methods for procuring N-SMase include

isolation of the protein from substantial quantities of urine. See, for example, S. Chatterjee, *J. Biol. Chem.*, 264(21):12554 (1989).

It thus would be desirable to have a convenient source of N-SMases. It also 5 would be desirable to have agents that can modulate N-SMase activity. It would be further desirable to have effective assays for identifying compounds that have the potential to modulate N-SMase activity or to diagnose or treat disorders relating to N-SMase.

## 10 SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids that encode human neutral sphingomyelinases (N-SMase) and N-SMase fragments and derivatives capable of hybridizing to such N-SMase-encoding nucleic acids. cDNA (SEQ ID NO:1) encoding human N-SMase has been isolated and expressed to provide recombinant N- 15 SMase having an apparent molecular weight of 44 kDa.

The invention further provides isolated recombinant human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives.

20 The invention also provides novel assays for identifying compounds useful in the diagnosis or treatment of human neutral sphingomyelinase related disorders. Preferred compounds identified through assays of the invention can modulate, particularly inhibit, human neutral sphingomyelinase activity.

25 A variety of such assays are provided including, e.g., cleavage assays, direct binding assays, as well as assays that identify a particular domain function. A preferred assay of the invention comprises providing 1) an isolated human neutral sphingomyelinase or a fragment or derivative thereof, 2) a human neutral sphingomyelinase cleavage target such as sphingomyelin, and 3) a candidate 30 pharmacological agent potentially useful in the diagnosis or treatment of disease

associated with human neutral sphingomyelinase, which agents 1), 2) and 3) are typically assayed in admixture. Those agents are suitably treated under conditions whereby, but for the presence of the candidate pharmacological agent, the N-SMase or fragment or derivative thereof selectively cleaves the cleavage target to yield a cleavage product such as ceramide. The agents are then analyzed for the presence of the cleavage product, wherein the absence or reduced concentration (e.g. relative to control, i.e. same mixture of agents 1) and 2) but without the candidate agent 3)) of the cleavage product indicates that the candidate pharmacological agent is capable of modulating N-SMase activity, particularly inhibition of sphingomyelin cleavage activity.

Agents identified through assays of the invention will have potential for use in a number of therapeutic applications, especially to modulate, particularly inhibit, expression or activity of human neutral sphingomyelinase in particular cells. Specific disorders that potentially could be treated by administration of pharmacological agents identified through assays of the invention include inflammatory disorders such as arthritis and osteroarthritis, treatment of obesity and diabetes, treatment of malignancies, and treatment of HIV. Identified agents also may be useful for treatment of cirrhosis of the liver and other liver diseases, to increase human plasma low density lipoproteins receptors (to thereby reduce excessive cholesterol levels of a subject), and for treatment of atherosclerosis.

Identified agents also may be useful for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage. Identified agents also may be useful to treat or inhibit undesired vascular restenosis e.g. subsequent to arterial plaque removal. Identified agents also may be useful in the treatment of central nervous system disorders such as treatment of depression, schizophrenia and Alzheimer's disease, and treatment or prevention or inhibition of neurodegeneration. Identified agents also may be useful to

- prevent transmission of malaria by application of the agent to areas frequented by malaria carriers to thereby prevent development of the parasite plasmodium.

The invention further provides methods to modulate expression or activity of N-SMase in particular cells through administering to a patient in need thereof a therapeutically effective amount of human neutral sphingomyelinase or N-SMase fragment or derivative thereof or a nucleic acid encoding same. Preferably, an N-SMase fragment or derivative or corresponding nucleic acid is administered that contains only selected domains to thereby modulate N-SMase activity as desired and without effects associated with the deleted or otherwise altered domain(s). For instance, as discussed in more detail below, it will be generally preferred the TSLKVPA domain of N-SMase or corresponding nucleic acid sequence will not be present in functional form in administered peptides or nucleic acids.

Such therapeutic methods can be employed to treat subjects susceptible to (i.e. prophylactic treatment) or suffering from N-SMase related disorders including e.g. inflammatory disorders such as arthritis and osteroarthritis, Crohn's disease, treatment of obesity and diabetes, treatment of malignancies, particularly cancers including susceptible solid tumors, treatment of HIV, treatment of renal failure, and to prevent or inhibit transmission of malaria. N-SMase or fragments or derivatives thereof, and nucleic acids encoding same of the invention, also can be used for treatment of cirrhosis of the liver and other liver diseases, and to increase human plasma low density lipoproteins (LDL) receptors and to thereby reduce excessive cholesterol levels. N-SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for treatment of atherosclerosis.

N-SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage. In this aspect, the invention provides sperm or seminal fluid in combination with an N-SMase

65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80

fragment or derivative to thereby provide enhanced viability or lifetime of samples during the storage period. The sperm or seminal fluid sample may be human, or of other mammal such as horse, cattle or other livestock.

5 N-SMase or fragments or derivatives, and nucleic acids encoding same, can be further employed to treat or inhibit undesired vascular restenosis e.g. subsequent to arterial plaque removal, and the treatment of central nervous system disorders such as treatment of depression, schizophrenia and Alzheimer's disease, and treatment or prevention or inhibition of neurodegeneration.

10

N-SMase or fragments or derivatives, and nucleic acids encoding same, particularly fragment or derivatives and nucleic acids that inhibit N-SMase activity, also may be administered to treat a patient, particularly a human, suffering from or susceptible to cardiac disease where LV dysfunction occurs, including a patient, 15 particularly a human, suffering from or susceptible to heart failure.

Other aspects of the invention are disclosed *infra*.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 shows a nucleotide sequence (SEQ ID NO:1) of isolated cDNA encoding human N-SMase.

Figure 2 shows the deduced amino acid sequence (SEQ ID NO:2) of human N-SMase. ♦ N-glycosylation site, • tyrosine kinase phosphorylation site, ♣ protein kinase phosphorylation sites, ♥ casein kinase II phosphorylation sites, ▽ cyclic AMP and cyclic GMP dependent protein phosphorylation sites, underline: myristylation sites, \* stop codon.

Figure 3 shows the hydropathy plot analysis of N-SMase.

30

Figures 4A and 4B show gel electrophoretic analysis of recombinant N-SMase expressed in *E. coli*. Figure 4A shows a Comassie blue staining and Figure 4B shows Western immunoblot analysis.

5 Figure 5 shows transient expression of recombinant N-SMase in Cos-7 cells.

Figure 6A and 6B show Northern blot analyses of N-SMase. Figure 6A shows tissue distribution of N-SMase. Figure 6B shows transcript size of human kidney N-SMase.

10

Figure 7 shows N-SMase activity in aortic smooth muscle cells transiently transfected with cDNA for N-SMase.

15 Figures 8A, 8B and 8C show chromatin condensation in transiently transfected aortic smooth muscle cells. Figure 8A shows control cells. Figure 8B shows PVS-SPOT. Figure 8C shows PHH1.

#### DETAILED DESCRIPTION OF THE INVENTION

We have now isolated cDNA encoding a human N-SMase. This cDNA is represented by SEQ ID NO:1 (Figure 1) and encodes a protein that when expressed in *E. coli* cells has an apparent molecular weight of 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarocine. That recombinant protein is bound by an antibody against the 92 kDa native N-SMase. Protein was also expressed in Cos-7 cells. The isolated and purified recombinant N-SMase has been shown to have N-SMase activity. See, for instance, the results disclosed in Example 1 which follows.

30 As discussed above, the ability to regulate N-SMase activity in a particular environment is very important. Too high a level of N-SMase or conversely too low a level of N-SMase can result in undesired effects. For example, excessively high N-

SMase levels can result in apoptosis, while excessively low levels of N-SMase can result in lack of cell proliferation or lack of LDL receptors. Similarly, N-SMase expression in one cell type can be desirable in leading to more efficient cholesterol processing, whereas its expression in another cell type can be undesirable.

5

As discussed above, the invention provides methods to modulate, including inhibition of, expression or activity of N-SMase in particular cells. For example, one can use a N-SMase nucleic acid segment operably linked to a N-SMase promoter to selectively direct it to desired cells. As another example, one can administer a N-SMase protein or fragment or derivative to modulate N-SMase activity.

10 The invention further provides isolated N-SMase having an amino acid sequence represented by SEQ ID NO:2 (Figure 2), as well as fragments or derivatives thereof.

15

The term "fragment" or "derivative" when referring to an N-SMase protein means proteins or polypeptides which retain essentially the same biological function or activity as the protein of SEQ ID NO:2. For example, the N-SMase fragments or derivatives of the present invention maintain at least about 50% of the activity of the protein of SEQ ID NO:2, preferably at least 75%, more preferably at least about 95% of the activity of the protein of SEQ ID NO:2, as determined e.g. by a standard activity gel assay such as the assay disclosed in Example 1, part 6, which follows and includes measuring activity of the N-SMase peptide using [<sup>14</sup>C]-sphingomyelin.

20 25 Fragments or derivatives as the term is used herein can include competitors of the native N-SMase with respect to a particular N-SMase domain activity. However, the fragment or derivative shows an overall similarity to N-SMase in other areas as explained herein.

An N-SMase fragment or derivative of the invention may be (i) a peptide in

- which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or

5 (ii) a peptide in which one or more of the amino acid residues includes a substituent group, or (iii) a peptide in which the mature protein is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol). Thus, an N-SMase fragment or derivative includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active

10 mature polypeptide.

The protein fragments and derivatives of the invention are of a sufficient length to uniquely identify a region of N-SMase. N-SMase fragments and derivatives thus preferably comprise at least 8 amino acids, usually at least about 12 amino acids,

15 more usually at least about 15 amino acids, still more typically at least about 30 amino acids, even more typically at least about 50 or 70 amino acids. Preferred N-SMase fragments or derivatives of the invention include those that have at least about 70 percent homology (sequence identity) to the protein of SEQ ID NO:2, more preferably about 80 percent or more homology to the protein of SEQ ID NO:2, still more

20 preferably about 85 to 90 percent or more homology to the protein of SEQ ID NO:2.

N-SMase and fragments and derivatives thereof of the invention are "isolated", meaning the protein or peptide constitutes at least about 70%, preferably at least about 85%, more preferably at least about 90% and still more preferably at least about 95% by weight of the total protein in a given sample. A protein or peptide of the invention preferably is also at least 70% free of immunoglobulin contaminants, more preferably at least 85% free, still more preferably at least 90% free and even more preferably at least 95% free of immunoglobulin contaminants. The N-SMase fragments and derivatives may be present in a free state or bound to other components, e.g. blocking groups to chemically insulate reactive groups (e.g. amines, carboxyls, etc.) of the

peptide, or fusion peptides or polypeptides (i.e. the peptide may be present as a portion of a larger polypeptide).

As discussed above, N-SMase nucleic acid fragments and derivatives are also provided. Those fragments and derivatives are of a length sufficient to bind to the sequence of SEQ ID NO:1 under the following moderately stringent conditions (referred to herein as "normal stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.8M saline/0.08M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing once with that SSC buffer at 37°C.

Preferred N-SMase nucleic acid fragments and derivatives of the invention will bind to the sequence of SEQ ID NO:1 under the following highly stringent conditions (referred to herein as "high stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer at a temperature of 42°C and remaining bound when subject to washing twice with that SSC buffer at 42°C.

These nucleic acid fragments and derivatives preferably should comprise at least 20 base pairs, more preferably at least about 50 base pairs, and still more preferably a nucleic acid fragment or derivative of the invention comprises at least about 100, 200, 300, 400, 500 or 800 base pairs. In some preferred embodiments, the nucleic acid fragment or derivative is bound to some moiety which permits ready identification such as a radionucleotide, fluorescent or other chemical identifier.

N-SMase will have a number of functional domains, e.g., a TNF- $\alpha$  55kDa receptor/Fas Apo(o)-1 domain, the sterol regulator element binding protein (SREBP) domain, etc. N-SMase of Figure 2 also has a domain (TSLKVPA, residues 258-264 of Figure 1; SEQ ID NO:3) homologous to the Staphylococcal enterotoxin-B peptide domain (RSITVRV; SEQ ID NO:4), which domain has been reported to elicit toxic

effects in human kidney cells. Accordingly, preferred N-SMase fragments and derivatives do not include that TSLKVPA domain in functional form. Other N-SMase domains can be readily identified by standard techniques such as deletion analysis.

5 In a similar manner, one can readily identify a deletion, addition or substitution that will inactivate a particular domain, e.g. by simply testing a fragment or derivative with altered domain to determine if the fragment or derivative exhibits activity associated with the altered domain. Any of a variety of tests can be employed, such as e.g. the *in vitro* tests of Example 1 which follows.

10

Thus, an N-SMase protein or nucleic acid fragment or derivative can be employed that contains only specific domains and can be administered to a subject such as a mammal to modulate N-SMase activity in targeted cells as desired.

15

Preferred N-SMase protein and nucleic acid fragments and derivatives include at least one functional domain region, e.g. the TNF- $\alpha$  binding domain, the SREBP domain or the sphingomyelin cleavage domain. Particularly preferred fragments and derivatives comprise one or more conserved peptides or corresponding nucleic acid sequences of at least one functional domain region.

20

Several apparent isoforms of human N-SMase exist that can be distinguished based on physical-chemical criteria, including electrophoretic migration rates, reactions against antibody of N-SMase and reactions to metals including copper, lithium and magnesium. The N-SMase and fragments and derivatives thereof of the present invention, and nucleic acids encoding same, include such isoforms.

30 Isolated N-SMase and peptide fragments or derivatives of the invention are preferably produced by recombinant methods. See the procedures disclosed in Example 1 which follows. A wide variety of molecular and biochemical methods are available for generating and expressing the N-SMase of the present invention; see e.g.

the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., - Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that 5 are otherwise known in the art. For example, N-SMase or fragments thereof may be obtained by chemical synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the N-SMase or fragment. The use of mammalian-based expression systems, particularly human, is particularly preferred 10 where the peptide is to be used therapeutically.

15 Nucleic acids encoding the novel N-SMase of the present invention and fragments and derivatives thereof may be part of N-SMase expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for disease associated with expression of a N-SMase), etc. Nucleic acids encoding N-SMase containing proteins are isolated from eukaryotic cells, preferably human cells, by screening cDNA libraries with probes or PCR primers derived from the disclosed N-SMase cDNAs.

20 The nucleic acids of the present invention are isolated, meaning the nucleic acids comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome and usually constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid 25 present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

The nucleic acids of the present invention find a wide variety of applications including: use as translatable transcripts, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of N-SMase genes and gene transcripts; use in detecting or amplifying nucleic acids encoding additional N-SMase homologs and structural analogs; and use in gene therapy applications.

For example, N-SMase nucleic acids can be used to modulate cellular expression or intracellular concentration or availability of active N-SMase. Thus, for example, N-SMase has been shown to be involved in liver cell proliferation (Alessenko and Chatterjee, *Mol. Cell. Biochem.* 143:119-174, (1995)), and thus N-SMase nucleic acids may be used to treat liver diseases such as cirrhosis.

To inhibit N-SMase activity, nucleic acid encoding a competitor or an antagonist can be administered to a subject. One preferred embodiment employs nucleic acid encoding an N-SMase derivative that acts as a competitor or an antagonist. For example, dependent upon the N-SMase activity desired to be inhibited, the N-SMase domain responsible for that activity can be appropriately altered (e.g. deleted or mutated), whereby the protein will still display the desired activity, but will not exhibit the undesired activity. Moreover, the altered protein can compete with the native N-SMase to thereby inhibit the undesired activity.

Further, to reduce N-SMase activity, nucleic acids capable of inhibiting translation of N-SMase also may be administered. These nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed relevant N-SMase fragment-encoding nucleic acid. Antisense modulation of the expression of a given N-SMase fragment containing protein may employ N-SMase fragment antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a vector comprising an N-SMase fragment sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous N-SMase fragment containing protein encoding

mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a given N-SMase fragment containing protein may be administered 5 to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in expression of the N-SMase.

The N-SMase nucleic acids are introduced into the target cell by any method which will result in the uptake and expression of the nucleic acid by the target cells. 10 These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a 15 fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical 20 conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem.*, 64:487 (1995); F. Lim et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); 25 A.I. Geller et al., *Proc Natl. Acad. Sci.: U.S.A.*:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet.*, 3:219 (1993); Yang et al., *J. Virol.*, 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA,  $\text{Ca}_3(\text{PO}_4)_2$  precipitation, DEAE dextran, electroporation, protoplast fusion, lipofection, cell microinjection, and viral vectors.

The vector can be employed to target essentially any desired target cell, such as a glioma. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Additionally, the particles can be delivered by intracerebroventricular (icv) infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA*, 91:2076-2080 (1994); Morrison et al., *Am. J. Physiol.*, 266: 292-305 (1994)). Other methods that can be used include catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, and oral or other known routes of administration.

The invention provides efficient screening methods to identify pharmacological agents or lead compounds for agents which modulate, e.g. interfere with or increase an N-SMase activity. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of pharmaceutical drug development programs.

A wide variety of assays are provided including, e.g., cleavage assays, direct binding assays as well as assays to identify a particular domain function.

A preferred assay mixture of the invention comprises at least a portion of the 5 N-SMase capable of cleaving a N-SMase cleavage target, e.g. sphingomyelin. An assay mixture of the invention also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different candidate agent concentrations to obtain a differential response to the various concentrations. Typically, one of these assay mixtures serves as a negative control, i.e. at zero 10 concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds and preferably small organic compounds. Small organic compounds suitably may have e.g. a molecular weight of more than about 50 yet less than about 2,500. Candidate agents may comprise functional chemical groups that interact with proteins and/or 15 DNA.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds 20 and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced.

Additionally, natural and synthetically produced libraries and compounds are 25 readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc.

65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

A variety of other reagents may also be included in the mixture. These include reagents such as salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the N-SMase or fragment or derivative thereof cleaves the cleavage target. The mixture components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4° and 40°C, more commonly between 15° and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high throughput screening, and are typically between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of the cleavage product is detected by any convenient way. For cell-free type assays, the cleavage target may be bound to a solid substrate and the cleavage product labelled, e.g., radiolabelled. A separation step can be used to separate the bound target from unbound cleavage product. The separation step may be accomplished in a variety of ways known in the art. The solid substrate may be made of a wide variety of materials and in a wide variety of shapes, e.g. microtiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, to minimize background binding, to facilitate washing and to minimize cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead (e.g. beads with iron cores may be readily isolated and washed using magnets),

particle, chromatographic column or filter with a wash solution or solvent. Typically,

- the separation step will include an extended rinse or wash or a plurality of rinses or

washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those

5 components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc. may exploit a polypeptide specific binding reagent such as an antibody or receptor specific to a ligand of the polypeptide.

As mentioned, detection may be effected in any convenient way, and for

10 cell-free assays, one of the components usually comprises or is coupled to a label.

Essentially any label can be used that provides for detection. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to a reagent or incorporated into the peptide structure, e.g. in the case of a peptide

15 reagent, a methionine residue comprising a radioactive isotope of sulfur.

A variety of methods may be used to detect the label depending on the nature

of the label and other assay components. For example, the label may be detected

bound to the solid substrate or a portion of the bound complex containing the label

20 may be separated from the solid substrate, and thereafter the label detected. Labels

may be directly detected through optical or electron density, radiative emissions,

nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

For example, in the case of radioactive labels, emissions may be detected directly,

e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters.

25

The assays of the invention are particularly suited to automated high

throughput drug screening. In a particular embodiment, an automated mechanism,

e.g. a mechanized arm, retrieves and transfers a microtiter plate to a liquid dispensing

station where measured aliquots of each of an incubation buffer and a solution

30 comprising one or more candidate agents are deposited into each designated well. The

arm then retrieves and transfers to and deposits in designated wells a measured aliquot of a solution comprising a N-SMase protein or fragment or derivative thereof as well as solutions of other reagents such as a cleavage target. Thereafter, the arm transfers the microtiter plate to an analysis station where the reaction mixture can be analyzed 5 for the presence or absence of various reaction products.

A preferred assay is disclosed in Example 2 which follows and which includes application of labeled sphingomyelin ( $[^{14}\text{C}]$ sphingomyelin) to a solid substrate such as a multi-well tray together with N-SMase.

10

As discussed above, N-SMase or fragments or derivatives thereof, and nucleic acids encoding same, also may be used for *in vitro* fertilization applications, particularly to improve viability and/or effective lifetime of sperm or seminal fluid samples during storage.

15

In this aspect, the invention also provides stored samples of human or other mammal such as cattle or horse sperm or seminal fluid in combination with an N-SMase fragment or derivative to thereby provide enhanced viability or lifetime of samples during the storage period.

20

For example, a sperm or seminal fluid storage unit of the invention may suitably comprise an N-SMase fragment or derivative of the invention in combination with a sperm or seminal fluid sample. That mixture also may optionally comprise a buffer or diluent as may be used with such samples. The N-SMase fragment or 25 derivative preferably will be present in an amount sufficient to enhance the viability of lifetime of the sperm or seminal fluid sample during the storage period. Such storage effective amount amounts can be readily determined empirically for the particular N-SMase fragment or derivative employed. Storage effective amounts suitably may be at least about 0.01 weight percent of N-SMase derivative or fragment thereof based on 30 total weight of the storage sample, more preferably at least about 0.05 weight percent.

50 55 60 65 70 75 80 85 90 95

The sperm or seminal fluid sample may be human or of other mammal, e.g. horse, cattle or other livestock sample. The storage unit may suitably be a sterile cryovial or other vessel as conventionally employed to store sperm and seminal fluid.

5 The proteins and nucleic acids and fragments and derivatives thereof of the invention also may be used to generate immune responses. For example, it has been found that in certain instances where inappropriate expression of a self-protein is occurring an immune reaction can be useful. The nucleic acid permits the creation of unique peptides that can generate such a reaction. The characteristics needed to 10 generate a peptide that will induce a MHC class I or II reaction are known and suitable peptides having such characteristics can be readily prepared based upon the present disclosure.

15 Antibodies also can be prepared that will bind to one or more particular domains of a peptide of the invention and can be used to modulate N-SMase activity.

Moreover, administration of an antibody against N-SMase or fragment or derivative thereof, preferably monoclonal or monospecific, to mammalian cells (including human cells) can reduce or abrogate TNF- $\alpha$  induced cell death (apoptosis) 20 and the invention includes such therapeutic methods. In such methods, antibody against N-SMase can be administered to a mammal (including a human) by known procedures. It has been specifically found that apoptosis of human leukemic (HL-60) cells expressing N-SMase was abrogated by treatment with antibody against N-SMase.

25 The preferred therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of N-SMase or fragment or derivative thereof, or nucleic acid encoding same, to an animal in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from or 30 susceptible to one of the above discussed disorders, including inflammatory disorders

such as arthritis, osteroarthritis and Crohn's disease, obesity, diabetes, malignancies, particularly cancers including susceptible solid tumors, HIV, liver disorders including cirrhosis, excessive cholesterol levels, renal failure, cholesteryl ester storage disorder, cardiac disease associated with LV dysfunction, atherosclerosis, undesired vascular restenosis, neurodegeneration, and central nervous system disorders such as depression, schizophrenia and Alzheimer's disease.

For therapeutic applications, peptides and nucleic acids of the invention may be suitably administered to a subject such as a mammal, particularly a human, alone or 10 as part of a pharmaceutical composition, comprising the peptide or nucleic acid together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

15 The pharmaceutical compositions of the invention include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes, and may be prepared by any methods 20 well known in the art of pharmacy. See, for example, *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA (17th ed. 1985).

Such preparative methods include the step of bringing into association with the molecule to be administered ingredients such as the carrier which constitutes one or 25 more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets optionally may be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; and pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

65 66 67 68 69 60 61 62 63 64

Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

- Application of the subject therapeutics often will be local, so as to be
  - 5 administered at the site of interest. Various techniques can be used for providing the subject compositions at the site of interest, such as injection, use of catheters, trocars, projectiles, pluronic gel, stents, sustained drug release polymers or other device which provides for internal access. Where an organ or tissue is accessible because of
    - 10 removal from the patient, such organ or tissue may be bathed in a medium containing the subject compositions, the subject compositions may be painted onto the organ, or may be applied in any convenient way. Systemic administration of a nucleic acid using lipofection, liposomes with tissue targeting (e.g. antibody) may also be employed.
- 15 It will be appreciated that actual preferred amounts of a given peptide or nucleic acid of the invention used in a given therapy will vary to the particular active peptide or nucleic acid being utilized, the particular compositions formulated, the mode of application, the particular site of administration, the patient's weight, general health, sex, etc., the particular indication being treated, etc. and other such factors
  - 20 that are recognized by those skilled in the art including the attendant physician or veterinarian. Optimal administration rates for a given protocol of administration can be readily determined by those skilled in the art using conventional dosage determination tests.
- 25 The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

## EXAMPLE 1 - Cloning and expression of human neutral Sphingomyelinase

## 1. Purification of human neutral sphingomyelinase and preparation of antibodies.

Neutral sphingomyelinase (N-SMase) was prepared from human urine and polyclonal antibodies against that enzyme were raised in rabbits as described previously by S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989). Monospecific polyclonal antibodies against N-SMase were prepared by appropriate immuno-affinity absorption techniques as described in *J. Biol. Chem.*, 264:12,534-12,561 (1989).

## 2. Screening of cDNA library.

The human kidney library was purchased from Clontech (Palo Alto, CA) and was screened using anti-N-SMase described above according to the manufacturers protocol. Briefly, the  $\lambda$ gt11 phage was plated at  $3 \times 10^4$  pfu/150 mm plate on a lawn of E.Coli strain y1090r. Incubation was carried out at 42°C for 3.5 hrs to allow lytic phage growth. Then, a filter saturated with 10 mM IPTG was placed on top of the plate and incubated overnight at 37°C. Next, the filter was blocked with a solution of 5% non-fat dry milk for 1 hr at room temperature. Next, the filter was incubated with antibody against N-SMase at 1:200 dilution at room temperature overnight, and signal was detected by enhanced chemiluminence technique (ECL, Amersham). Sixty-three clones were obtained by screening  $1 \times 10^6$   $\lambda$ gt11 phage clones. The most intense clones of cells were subjected to secondary and tertiary screening. All positive clones were subject to PCR to identify their insert size. Finally, a clone containing the longest insert (3.7 kb) referred to as  $\lambda^{32}$ -1 was used for further analysis by subcloning, sequencing and expression.

## 3. Preparation of fusion proteins.

The protocol as described by the manufacturer's manual (Biotech, #5, 1992, pg. 636) was followed. By this procedure, the host growing in the logarithmic phase

was infected with phage and incubated for 2 hr at 30°C. Next, IPTG (10 mM) was added and incubation continued at 37°C. The cell cultures were removed at 0, 15 min., 30 min., 45 min., 1 hr, 2 hr, 4 hr and 24 hr. The cells were centrifuged, washed with PBS, and stored frozen for N-SMase activity measurement.

5

4. Subcloning and sequencing of N-SMase cDNA.

First, the  $\lambda^{32}$ -1 DNA was purified with the Magical Lambda preps DNA purification system from Promega by following the manufacturer's manual. Then, it was digested with the restriction endonuclease EcoRI. The 3.7kb insert was gel purified and subcloned into the EcoRI site of the vector pBluescriptII-SK (Stratagene, La Jolla, CA). The plasmid, thus generated was termed pBC32-2, and was purified using QIAGEN's DNA purification system. The N-SMase cDNA insert was sequenced with Sequenase using T7 and T3 primers by automatic sequence machine Model 373A (Applied Biosystems).

15

5. Transient Expression of N-SMase in Cos-7 cells.

To put N-SMase cDNA into a transient expression vector, the pBC32-2 was double digested with restriction endonucleosidases NotI and Sal1. The 3.6 kb insert containing N-SMase was gel purified and inserted into a transient expression vector PSV-SPOT-1 (BRL). Thus, a plasmid called pH<sub>H1</sub> was constructed.

25 To transfect Cos-7 cells with pH<sub>H1</sub> and mock vector,  $3 \times 10^5$  Cos-1 cells/plate in a p100 plate in 8 ml of Dulbecco's modified Eagle's medium (D-MEM) were seeded with 10% FCS (Bethesda Research Laboratory; BRL). Cells were incubated in a 10% CO<sub>2</sub> 37°C incubator until they are 80% confluent. The cells were then transfected with 10  $\mu$ g of purified pH<sub>H1</sub> (QIAGEN) using Lipofectamine™ (BRL) in medium. Medium was changed after 5 hours of incubation. Finally, the cells were harvested at various time points (16 hr, 24 hr, 36 hr, and 48 hr, post transfection) by centrifugation at 1500 xg for 10 min., washed with phosphate buffered saline (PBS) 30 and stored frozen at -20°C.

## 6. Activity gel assay of N-SMase expression in transfected Cos-7 cells.

Both the bacterial cells and Cos-7 cells transfected with N-SMase cDNA were homogenized in Tris-glycine buffer (pH 7.4) containing 0.1% cutscum. The samples were mixed vigorously and sonicated for 10 sec. Next, the samples were transferred to a 4°C incubator and shaken for about 2 hours. Every hour, the samples were sonicated again on ice and further shaken. Subsequently, the samples were centrifuged at 10,000 xg for 10 min. The supernatants were collected, the protein content was measured and subjected to polyacrylamide gel electrophoresis using sodium laurylsarcosine. Subsequent to electrophoresis, the gel was sliced into several pieces and the activity of N-SMase was measured using [<sup>14</sup>C]-sphingomyelin as a substrate (see T. Taki and S. Chatterjee, *Analyt. Biochem.*, 224:490-493 (1995)).

## 7. Measurement of sphingomyelinase activity in aortic smooth muscle cells transiently transfected with N-SMase cDNA.

15 The activity of sphingomyelinase was measured in aortic smooth muscle cell extracts transiently transfected with pSVSPOT (mock cDNA) and pH1 (cDNA for N-SMase) as shown previously in P. Ghosh and S. Chatterjee, *J. Biol. Chem.*, 262:12,550-12,556 (1987)). Briefly, the cell extracts (100 µg protein) were incubated with cutscum, MgCl<sub>2</sub>, human serum albumin and Tris glycine buffer (pH 7.4) plus 20 [<sup>14</sup>C]-sphingomyelin (15,000 cpm) for 1 hr at 37°C. The reaction was terminated with 1 ml of 10% TCA and centrifuged. The supernatant was extracted with diethylether and the aqueous layer was withdrawn to measure radioactivity.

## 8. Reverse transcriptase-polymerase chain reaction.

25 Human kidney cells were seeded (1 x 10<sup>5</sup> cell/plate in p100 plates) and grown to confluence in complete medium with 10% fetal calf serum. Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction. 1 µg of total RNA was used to synthesize first strand cDNA using 20 pmol random hexamer primer, 200 units moloney murine leukemia virus reverse transcriptase, 0.5 mM each 30 of deoxynucleotide triphosphates (Clontech, Palo Alto, CA) in buffer containing 50

mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 0.5 units of RNase inhibitor, pH 8.3 at 42°C for 1 hour. 1  $\mu$ l of 1:100 dilution of cDNA products was used to run PCR in 50  $\mu$ l reaction mixture containing 0.2 mM each of dNTP, 2.0 units of Taq polymerase, 0.4 uM of each primers, 10 mM Tris HCl, 50 mM KCl, and 1.5 mM MgCl<sub>2</sub> (pH 8.3). The PCR was run for 35 cycles (30s at 94°C, 30s at 55°C and 30s at 72 °C) using a Perkin-Elmer Thermocycler.

9. Northern blot analysis.

One set of sequence primers from pBC32-2, T3-2/4T3R5, as RT-PCR primer was selected to conduct RT-PCR as described above. The 18-mer primers had the following sequence: TTGCGGCACTATTAGGTG (SEQ ID NO:5) and CGCCAATGCCAAAACGTA (SEQ ID NO:6). A 465 bp specific product was obtained, and gel purified. 50 ng of this product was labeled with 25  $\mu$ Ci[ $\alpha$ -<sup>32</sup>P] dATP and 25  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dCTP using Random hexamer primers (BRL). The specific activity of this probe was 1.88 x 10<sup>9</sup> cpm/ $\mu$ g. Next, the multiple tissue northern blot (Clontech; Palo Alto, CA) were hybridized with this probe(2x10) at 50°C overnight with hybridization buffer. Next, the blots were washed twice in 2x SSC, 0.05% SDS at room temperature for 30-40 min, then in 0.1 x SSC and 0.05% SDS for 40 min. at 50°C. Finally, the blot was exposed to an x-ray film at -70°C using two intensifying screens overnight.

10. Expression and purification of Glutathione-S-Transferase (GST)-NSMase fusion protein in E.Coli.

To prepare GST-NSMase fusion protein, an expression plasmid pJK2, pBC32-2 was digested with BssHII and EcoRI. A 2793 bp insert representing N-SMase open reading frame that is missing 18 bp N-terminal sequence, was ligated with a phosphorylated BamHI-BssHII linker containing the N-terminal sequence of GATCCATGATGACATATCACGAAACGCGCGTTCTGTGATA TGTCATCATG (SEQ ID NO:7) and a pGEX4T-1 vector double digested with BamHI and EcoRI (Phamacia; Piscataway, NJ). To express and purify GST-N-SMase fusion

protein, plasmid pJK2 was transformed into E.Coli (HB101) cells. A single colony of HB101 [pJK2] was grown in 2X YTA medium at 30°C until appropriate cell density (A600=1.5) was achieved. IPTG (0.1M) was added to induce fusion protein expression for 2 hours. Cells were harvested and the fusion protein was purified using Glutathione Sepharose-4B chromatography according to instructions provided by the manufacturer. N-SMase was released from the fusion protein by thrombin digestion. Such preparations were subjected to activity measurements and western immunoblot assays.

10 11. Coomassie blue staining and Western immunoblot assays of r-N-SMase. 20  $\mu$ g of purified r-N-SMase was subjected to electrophoresis on 12.5% SDS-PAGE gel. One gel was stained with Coomassie blue according to standard protocol. Another gel were transferred onto a PVDF membrane. Next, the polyvinylidiflouride (PVDF) membrane was blocked with 1% bovine serum albumin in TBS-T, incubated 15 with anti-N-SMase at a dilution of 1:200 and developed with horse radish peroxidase (see S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989)).

12. Measurement of apoptosis in aortic smooth muscle cells transfected with cDNA for N-SMase.

20 Aortic smooth muscle cells transiently transfected with PVSPOT (mock cDNA) and pH1 (cDNA for N-SMase) for 24 hours were solubilized and subjected to agarose gel electrophoresis (2 hr at 90 volts) for DNA fragmentation analysis.

25 Another set of cells transfected with cDNA were subjected to staining with the DNA-binding fluorochrome bis-benzimidine (Hoescht 323288; Sigma Chemical Co., St. Louis, MO). Briefly, transfected cells grown on glass cover slips were washed with PBS. The cells were fixed with 3% paraformaldehyde in PBS and incubated for 10 min at room temperature. The cells were washed with PBS and stained with 16  $\mu$ g/ml of bis-benzimidine in PBS. After 15 min of incubation at room temperature, 30 the samples were photographed. An Olympus BH<sub>2</sub> flourescence microscope with a

BH<sub>2</sub>-DMU U<sub>2</sub> UV mirror cube filter was used. Cells with three or more chromatin fragments were considered apoptotic.

The above-mentioned 3.7 kb nucleotide sequence of cDNA revealed an open reading frame size of 1197 base pairs which predicts a 397 amino acid polypeptide. The deduced amino acid sequence is shown in Figure 2. The estimated protein molecular weight is approximately 44 kDa, the estimated pI is 4.93. There are several potential modification sites in this protein: 1 N-glycosylation site at amino acid position 353; 1 tyrosine phosphorylation site at 238; and 2 cyclic AMP and cyclic GMP dependent protein kinase phosphorylation sites at position 218 and 357. It also has four casein kinase II phosphorylation sites at 3, 33, 65 and 101. Five myristylation sites at 28, 44, 205, 206 and 220 bases were also found. There are ten protein kinase-C phosphorylation sites at 38, 48, 164, 216, 217, 236, 251, 260, 323 and 355.

15

Hydropathy plot analysis of N-SMase (Figure 3 of the drawings) indicates that there is no apparent transmembrane domain. The N-glycosylation site at amino acid position 353, tyrosine phosphorylation site at 238, as well as several other phosphorylation sites are presumably located on the exterior. Such sites may be subjected to further glycosylation and phosphorylation.

To confirm that the 3.7 kb cDNA does encode N-SMase with an apparent molecular weight of 44 kDa, constructs were made to fuse cDNA coding region with Glutathione-S-Transferase. Then, this plasmid was transformed into *E.coli* (HB101) to express and purify GST-NSMase fusion protein. The expression of fusion protein was induced by IPTG (0.1M) for 2 hours. The fusion protein was purified using Glutathione Sepharose-4B chromatography. The fusion protein has an apparent molecular weight of 73 kDa. After thrombin digestion, it resolved into two bands, 29 kDa and 44 kDa which correspond to GST and N-SMase, respectively. After N-SMase was released from fusion protein by Glutathione Sepharose-4B

chromatography, it resolved as a single band, having a molecular weight on the order of 44 kDa. This protein was recognized by antibody against human N-SMase. See Figures 4A and 4B. Affinity-purified recombinant N-SMase expressed in *E.coli* had activity on the order of 3.9 nmole/mg protein/2h compared to mock cDNA transfected cells which had activity of 2.9 nmoles/mg protein/2h).

The results set forth in Figure 5 of the drawings show that Cos-7 cells transfected with pHII exhibited a 10-fold increase in N-SMase activity compared to cells transfected with mock vector pSPOT-1 24 hours post-transfection. The most active recombinant N-SMase had an apparent molecular weight of 100 kDa. Significant N-SMase activity in a descending order was observed in protein bands having apparent kDa of 130 and 74. Those results indicate that N-SMase undergoes multiple post-translational modification.

The 465 bp RT-PCR fragment amplified 5' end of N-SMase cDNA coding region was used to probe N-SMase mRNA in various human tissues. N-SMase was expressed in all the human tissues investigated, the transcript size and copy numbers varied from one tissue to another (see Figure 6A of the drawings). The major transcript size of N-SMase expressed in all of these tissues is 1.7 kb. The other transcripts are 900 bp, 400 bp and 200 bp. Extended exposures of the x-ray film showed additional transcripts on the order of 2.5 kb and 4.0 kb (Figure 6B) in human kidney. The cDNA of  $\lambda^{32}$ -1 consists of 3670 bp, and contains a polyadenylation signal (ATTATT) at 351, (ATTAAA) at 805, (AATTAA) at 2562 and (ATTAAA) at 2835. These polyadenylation signals vary from the consensus AATAAA sequence, but it was found in 12% (ATTAAA) and 2% (AATTAA, ATTATT) of the mRNAs in vertebrates, respectively. Such smaller transcripts exist due to their termination at different locations. The 1.7 kb transcript may be derived either from different genes or from alternative splicing.

Aortic smooth muscle cells transiently transfected with pHH1 (cDNA for N-SMase) had a 5-fold increase in enzyme activity compared to control (pSVSPOT) (see Figure 7). This was accompanied by DNA fragmentation in pHH1 transfected cells and chromatin condensation. Based on an analysis of about 500 cells, about 32% of the cells were found to be apoptotic (see Figure 8C) as compared to control (Figure 8A) and mock cDNA transfected cells (Figure 8B). Those findings indicate that over-expression of N-SMase in aortic smooth muscle cells is accompanied by an increase in enzyme activity and apoptosis.

10 EXAMPLE 2 - Protocol of a preferred N-SMase cleavage assay.

15 A. Reagents: N-methyl-<sup>14</sup>C]sphingomyelin (22,000 dpm/2  $\mu$ l in toluene:ethanol #2 v/v). Cutsum (detergent) 0.002%, MgCl<sub>2</sub>, 20  $\mu$ g human serum albumin, 25  $\mu$ Mol Tris-glycine buffer pH 7.4. Enzyme (neutral sphingomyelinase) (1 ng - 1  $\mu$ g/well).

20 B. Preparation of Assay Plates:

25 First 2  $\mu$ l of [<sup>14</sup>C]sphingomyelin (22,000 dpm) is applied at the center of the PVDF well (Millipore MAIP-S-45-10), high protein binding Immobilon-P (0.4  $\mu$  thick in a 96 well plastic tray. The assay plates are dried in vacuum and stored until use.

30 C. Assay:

The above reagents of Part A. are added to the well. Next, increasing concentration of the pure N-SMase (1 ng - 1  $\mu$ g) or samples of enzyme (human fluids, cell extracts, etc.) are added. Then, the sphingomyelinase assay (incubation at 37°C for 30 minutes) is conducted. The contents of the reaction mixture are removed by suction attached to the bottom of the 96 well tray. After washing with PBS (5 times/50  $\mu$ l) to remove non-specific radioactivity, 10  $\mu$ l of liquid scintillation cocktail is added to each well and the [<sup>14</sup>C]sphingomyelin radioactivity which remains on the PVDF 96 well tray is counted in a Packard top  $\beta$  counter.

This assay can be completely automated employing modern robotic systems.

- This assay also enables high-throughput, e.g. 96 samples can be conveniently assayed for sphingomyelinase in 30 minutes. The assay also enables screening or identifying both inhibitors (antagonists) and activators (agonists) of N-SMase. The assay also
- 5 may be employed to assay any enzyme that requires a lipid as a substrate that can be adsorbed to PVDF. The method also may be employed for antigen antibody binding assays, receptors binding assays, bacterial assays, viral assays and other toxin binding assays.

10 D. Controls for Assays:

Purified N-Smase (1 ng - 1  $\mu$ g) is used as a standard control. Additionally, a constant set of samples (human urine) spiked with pure N-SMase are preferably employed and activity of such samples measured to serve as quality control and to assess day-to-day variation in the results.

15

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

2025 RELEASE UNDER E.O. 14176

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: Chatterjee, Subroto

(ii) TITLE OF THE INVENTION: RECOMBINANT N-SMASES AND NUCLEIC ACIDS  
ENCODING SAME

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
- (B) STREET: 130 Water Street
- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Corless, Peter F
- (B) REGISTRATION NUMBER: 33,860
- (C) REFERENCE/DOCKET NUMBER: 46906

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 617-523-3400
- (B) TELEFAX: 617-523-6440
- (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1197 base pairs
- (B) TYPE: nucleic acid

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGATGACAT	ATCACGAAAC	GCGCGCGTTG	GCTCAAAGCG	ACTTACAGCA	ACTCTATGCG	60
GCACATTGAAA	CAACTGAATT	TGGCGCTTAC	TTTGCACAC	CCGCTGATGA	TACTTTACGT	120
TTTGGCATTG	GCGCAATCGC	TACGGCAAAA	ACGGCTCAGG	CATTACAAGG	TGCGGTTGTT	180
TTTGGTGCAC	AGTCATTGA	TGAACAAGAG	TACCCGCAGT	CTGAATTGAT	GGCGGGTTTT	240
TGGTTTGTCC	CCGAAGTGAT	GGTGACCATC	CGGGCAGATA	AAATCACGTT	CGGATCAGAT	300
ACCGTATCTG	ATTTTACGAC	GTGGCTGGCG	CAGTTCGTGC	AAAACAGCC	AAATACGGTG	360
ACCACTAGTC	ATGTGACAGA	TGAAGTGGAT	TGGATCGAAC	GGACAGAGAA	TTTGATTGAT	420
ACCTTAGCCA	TCGATCAAAC	CTTAGCCAAA	GTCGTTTTG	GTCGGCAACA	GACCCTGCAG	480
TTATCCGACA	CGTTACGACT	GGCACAAATT	ATTCGTGCGT	TAGCTGAGCA	GGCGAATACG	540
TATCATGTGG	TTTTAAAGCG	ACATGATGAA	TTGTTTATTT	CAGCAACACC	GGAACGGTTA	600
GTGGCTATGT	CAGGTGGTCA	GATCGCTACG	GC GGCGGTGCG	CTGGGACAAG	CCGGCGCGGG	660
ACGGATGGCG	CTGACGATAT	CGCGTTAGGC	GAAGCGTTGT	TAGCCAGTCA	GAAAAACCGC	720
ATTGAACATC	AATATGTCGT	GGCAAGTATC	ACGACACGCT	TGCAAGACGT	GACGACGTCG	780
CTAAAGGTGC	CGGCCATGCC	AAGTTTACTC	AAAAATAAGC	AAGTCAGCA	TTTGTACACA	840
CCAATTACAG	GGGACATTGC	GGCACATTAA	AGTGTGACCG	CGATTGTTGA	CCGCTTGCAT	900
CCAACACCAAG	CACTGGGTGG	CGTCCCACGT	GAAGCGGCC	TGTATTACAT	TGCGACCCAT	960
GAGAAGACAC	CTCGTGGCTT	GTTCGAGGT	CCTATTGGCT	ATTTTACCGC	AGATAATAGT	1020
GGGAATTG	TGGTTGGCAT	CCGTTCCATG	TATGTGAATC	AAACGCAGCG	ACGAGCAACT	1080
TTATTTGCTG	GTGCCGGGAT	TGTGGCTGAC	TCCGATGCGC	AACAAGAATA	TGAAGAAACT	1140
GGGTTGAAAT	TTGAACCCAT	GCGGCAATTG	TTAAAGGACT	ACAATCATGT	CGAATGAA	1197

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 397 amino acids
  - (B) TYPE: amino acid
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Met	Thr	Tyr	His	Glu	Thr	Arg	Ala	Leu	Ala	Gln	Ser	Asp	Leu	Gln
1					5				10					15	
Gln	Leu	Tyr	Ala	Ala	Leu	Glu	Thr	Thr	Glu	Phe	Gly	Ala	Tyr	Phe	Ala
									20	25			30		
Thr	Pro	Ala	Asp	Asp	Thr	Leu	Arg	Phe	Gly	Ile	Gly	Ala	Ile	Ala	Thr
									35	40			45		
Ala	Lys	Thr	Ala	Gln	Ala	Leu	Gln	Gly	Ala	Val	Phe	Gly	Ala	Gln	Ser
									50	55			60		
Phe	Asp	Glu	Gln	Glu	Tyr	Pro	Gln	Ser	Glu	Leu	Met	Ala	Gly	Phe	Trp

65	70	75	80
Phe Val Pro Glu Val Met Val Thr Ile Ala Ala Asp Lys Ile Thr Phe			
85	90		95
Gly Ser Asp Thr Val Ser Asp Phe Thr Thr Trp Leu Ala Gln Phe Val			
100	105		110
Pro Lys Gln Pro Asn Thr Val Thr Thr Ser His Val Thr Asp Glu Val			
115	120		125
Asp Trp Ile Glu Arg Thr Glu Asn Leu Ile Asp Thr Leu Ala Ile Asp			
130	135		140
Gln Thr Leu Ala Lys Val Val Phe Gly Arg Gln Thr Leu Gln Leu			
145	150		160
Ser Asp Thr Leu Arg Leu Ala Gln Ile Ile Arg Ala Leu Ala Glu Gln			
165	170		175
Ala Asn Thr Tyr His Val Val Leu Lys Arg His Asp Glu Leu Phe Ile			
180	185		190
Ser Ala Thr Pro Glu Arg Leu Val Ala Met Ser Gly Gly Gln Ile Ala			
195	200		205
Thr Ala Ala Val Ala Gly Thr Ser Arg Arg Gly Thr Asp Gly Ala Asp			
210	215		220
Asp Ile Ala Leu Gly Glu Ala Leu Leu Ala Ser Gln Lys Asn Arg Ile			
225	230		240
Glu His Gln Tyr Val Val Ala Ser Ile Thr Thr Arg Leu Gln Asp Val			
245	250		255
Thr Thr Ser Leu Lys Val Pro Ala Met Pro Ser Leu Leu Lys Asn Lys			
260	265		270
Gln Val Gln His Leu Tyr Thr Pro Ile Thr Gly Asp Ile Ala Ala His			
275	280		285
Leu Ser Val Thr Ala Ile Val Asp Arg Leu His Pro Thr Pro Ala Leu			
290	295		300
Gly Gly Val Pro Arg Glu Ala Ala Leu Tyr Tyr Ile Ala Thr His Glu			
305	310		320
Lys Thr Pro Arg Gly Leu Phe Ala Gly Pro Ile Gly Tyr Phe Thr Ala			
325	330		335
Asp Asn Ser Gly Glu Phe Val Val Gly Ile Arg Ser Met Tyr Val Asn			
340	345		350
Gln Thr Gln Arg Arg Ala Thr Leu Phe Ala Gly Ala Gly Ile Val Ala			
355	360		365
Asp Ser Asp Ala Gln Gln Glu Tyr Glu Glu Thr Gly Leu Lys Phe Glu			
370	375		380
Pro Met Arg Gln Leu Leu Lys Asp Tyr Asn His Val Glu			
385	390		395

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

~ (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Leu Lys Val Pro Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Ile Thr Val Arg Val  
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGCGGGCACT ATTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (ii) MOLECULE TYPE:
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAATGCC AAAACGTA

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(ii) MOLECULE TYPE:

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCATGAT GACATATCAC GAAACGCGCG TTTCGTGATA TGTCAATCATG

50

卷之三

What is claimed is:

1. An isolated nucleic acid encoding human neutral sphingomyelinase.
2. The nucleic acid of claim 1 where the nucleic acid comprises the sequence of SEQ ID NO:1, or the complement thereto.
3. The nucleic acid of claim 1 where the nucleic acid codes for the human neutral sphingomyelinase of SEQ ID NO:2.
4. The nucleic acid of claim 1 where the human neutral sphingomyelinase has a molecular weight of about 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarocine.
5. The nucleic acid of claim 1 where the nucleic acid has at least about 80 percent sequence identity to SEQ ID NO:1, or the complement thereto.
6. The nucleic acid of claim 1 wherein the polynucleotide is cDNA.
7. The nucleic acid of claim 1 wherein the polynucleotide is RNA.
8. A recombinant vector comprising the nucleic acid of claim 1.
9. A host cell comprising the vector of claim 8.
10. A method of producing human neutral sphingomyelinase comprising culturing a host cell of claim 9 under conditions suitable for expression of human neutral sphingomyelinase.

11. A nucleic acid that hybridizes to the sequence of SEQ ID NO:1 under normal stringency conditions.

12. The nucleic acid of claim 11 where the nucleic acid hybridizes to the sequence of SEQ ID NO:1 under high stringency conditions.

13. A method of identifying a compound useful in the diagnosis or treatment of a human neutral sphingomyelinase related disorder, comprising contacting a candidate pharmacological agent with human neutral sphingomyelinase or fragment or derivative thereof and analyzing the mixture of the candidate agent and human neutral sphingomyelinase or fragment or derivative thereof.

14. The method of claim 13 wherein the human neutral sphingomyelinase has a sequence represented by SEQ ID NO:2.

15. The method of claim 13 wherein

- 1) a mixture is formed of i) a human neutral sphingomyelinase cleavage target, ii) the human neutral sphingomyelinase or fragment or derivative thereof, and iii) a candidate pharmacological agent;
- 2) the mixture is treated under conditions whereby, but for the presence of the candidate agent, the human neutral sphingomyelinase or fragment or derivative cleaves the cleavage target to yield a cleavage product; and
- 3) the presence of the cleavage product is detected, wherein a reduced concentration of the cleavage product relative to a control mixture that does not contain the candidate agent identifies the candidate agent as a compound potentially useful in the diagnosis or treatment of a human neutral sphingomyelinase related disorder.

16. The method of claim 15 wherein the human neutral sphingomyelinase cleavage target is sphingomyelin.

17. The method of claim 15 wherein the human neutral sphingomyelinase cleavage product is ceramide.

18. An isolated human neutral sphingomyelinase having an apparent molecular weight of about 44 kDa as determined by polyacrylamide gel electrophoresis using sodium laurylsarocene.

19. An isolated human neutral sphingomyelinase of claim 18 comprising a sequence represented by SEQ ID NO:2.

20. An isolated polypeptide having at least about 70 percent sequence identity to SEQ ID NO:2.

21. A method for modulating N-SMase activity comprising administering to human cells a modulation effective amount of a nucleic acid of claim 1 or fragment or derivative thereof.

22. A method for modulating N-SMase activity comprising administering to human cells a modulation effective amount of an isolated human neutral sphingomyelinase of claim 18 or fragment or derivative thereof.

23. A method for treating a disorder associated with N-SMase comprising administering to a patient suffering from or susceptible to such disorder an effective amount of an isolated nucleic acid of claim 1 or fragment or derivative thereof.

24. The method of claim 23 wherein the disorder is an inflammatory disorder, arthritis, osteroarthritis, Crohn's disease, obesity, diabetes, cirrhosis, susceptible tumors, central nervous system disorder, vascular restonsis, arterial occlusion arising from plaque formation, cardiac disease where LV dysfunction

60 59 58 57 56 55 54 53 52 51 50

occurs, hyperchloesterolemia, cholestryl ester storage disorder, renal failure, HIV infection, depression, schizophrenia, neurodegeneration and Alzheimer's disease.

25. A method for treating a disorder associated with N-SMase comprising administering to a patient suffering from or susceptible to such disorder an effective amount of an isolated human neutral sphingomyelinase of claim 18 or fragment or derivative thereof.

26. The method of claim 25 wherein the disorder is an inflammatory disorder, arthritis, osteoarthritis, Crohn's disease, obesity, diabetes, cirrhosis, susceptible tumors, central nervous system disorder, vascular restonsis, arterial occlusion arising from plaque formation, cardiac disease where LV dysfunction occurs, hyperchloesterolemia, cholestryl ester storage disorder, renal failure, HIV infection, depression, schizophrenia, neurodegeneration and Alzheimer's disease.

27. A method of maintaining samples of sperm or seminal fluid comprising providing a mixture comprising sperm or seminal fluid and an effective amount of an a fragment or derivative of human neutral sphingomyelinase.

28. The method of claim 27 wherein the mixture comprises human sperm or seminal fluid.

29. A storage sample of sperm or seminal fluid comprising sperm or seminal fluid and a storage effective amount of a fragment or derivative of human neutral sphingomyelinase of claim 18.

30. A method to reduce TNF- $\alpha$  induced apoptosis of mammalian cells comprising administering to a mammal an effective amount of antibody against N-SMase or fragment or derivative thereof.

**ABSTRACT**

Isolated nucleic acids are provided that encode human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives capable of hybridizing to such N-SMase-encoding nucleic acids. The invention also includes isolated recombinant human neutral sphingomyelinase (N-SMase) and N-SMase fragments and derivatives are also provided. Novel assays are also provided to identify compounds useful in the diagnosis or treatment of human neutral sphingomyelinase related disorders.

65 12 30 62 32 24 60

ATGATGACATAATCACGAAACCGCGCGCG TTGGCTCAAAGCGACTTACAGCA  
ACTCTATGCGGCACTTGAAACAAC TGAAATTGGCGCTTA  
CTTTGCGACACCCGCTGATGATAC TTTACGTTTGGCATTGGCGCAATCG  
CT ACGGGCAAAACGGCTCAGGCATTACAAGGTGGGTTGGCATTGGCGCAATCG  
CAGTCATTGATGAAACAAGAGTACCCCGCAGTCTGAATTGATGGCGGGGTT  
TTGGTTTGTCCCCGAAAGTGTGATGGTACCCGGCAAGATAAAATCACGT  
TGGGATCAGATAACCGTATCTGATTACGGACGTGGCTGGCGCAGTTCGTG  
CCAAAACAGCCAAATACGGTACCACTAGTCATGTGACAGATGAAGTGGG  
TTGGATCGAACCGGACAGAGAATTGATTGATGATAACCTTAGCCATCGATCAA  
CCTTAGCCAAGTGGTTTGGTCCGGCAACAGAACCCCTGCAGTTATCCGAC  
ACGTTACGGACTGGCACAAATTATTGGCTGGGTTAGCTGAGCAGGGAAATAC  
GTATCATGTTGTTAAAGCGACATGATGAATTGTTATT CAGCAACAC  
CGGAACGGTTAGTGGCTATGTCAAGGTGGTCAGATCGCTACGGCGGGTC  
GCTGGGACAAGCCGGCGCGGACGGATGGCGCTGACGGATATCGCGTTAGG  
CGAAGCGTTGTTAGCCAGTCAAGAAAACCGCATTGAACATCAATA TGTG  
TGGCAAGTATCACCGACACGGCTTGCAAGACGTGACGGACGGTGGCTAAAGGTG  
CGGCCATGCCAAGTTTACTCAAAAATAAGCAAGTTCAAGCATTGTAACAC  
ACCAAATTACAGGGGACATTGGGCACATTAAAGTGTGACCGCGATTGTTG  
ACCGCTTGCACTCAACACCAAGCAGCACTGGGTGGCTGGCTGGCTAAAGGGCC  
CTGTATTACATTGCGACCCATGAGAAAGACACCTCGTGCTTGTGTTGGCA  
TCCTATTGGCTATTACCGCAGATAATAGTGGGGAAATTGGGGTGGCT  
TCCGTTCCATGTTATGTAATCAAACCGGAGCGACGAGCAACTTATTGCT  
GGTGCCTGGGATTGGGCTGACTCCGATGGCAACAAGAATATGAAGAAC  
TGGTTGAAAATTGAACCCATGCGGCAATTGTTAAAGGACTACAATCATG  
TGAATGA

FIG. 1

1 M M T Y H E T R A L A Q S D L Q Q L Y A A L E T T E F G A Y F A T P A  
— ♦  
36 D D T L R F G I G A I A T A K T A Q A L Q G A V F G A Q S F D E Q E Y  
♦ ♦  
71 P Q S E L M A G F W F V P E V M V T I A A D K I T F G S D T V S D F T  
106 T W L A Q F V P K Q P N T V T T S H V T D E V D W I E R T E N L I D T  
♣ ♦  
141 L A I D Q T L A K V V F G R Q Q T L Q L S D T L R L A Q I I R A L A E  
176 Q A N T Y H V V L K R H D E L F I S A T P E R L V A M S G G Q I A T A  
V  
211 A V A G T S R R G T D G A D D I A L G E A L L A S Q K N R I E H Q Y V  
♦ ♦ ♦  
246 V A S I T T R L Q D V T T S L K V P A M P S L L K N K Q V Q H L Y T P  
281 I T G D I A A H L S V T A I V D R L H P T P A L G G V P R E A A L Y Y  
♦ ♦  
316 I A T H E K T P R G L F A G P I G Y F T A D N S G E F V V G I R S M Y  
♦ ♦ V  
351 V N Q T Q R R A T L F A G A G I V A D S D A Q Q E Y E E T G L K F E P  
385 M R Q L L K D Y N H V E

FIG. 2

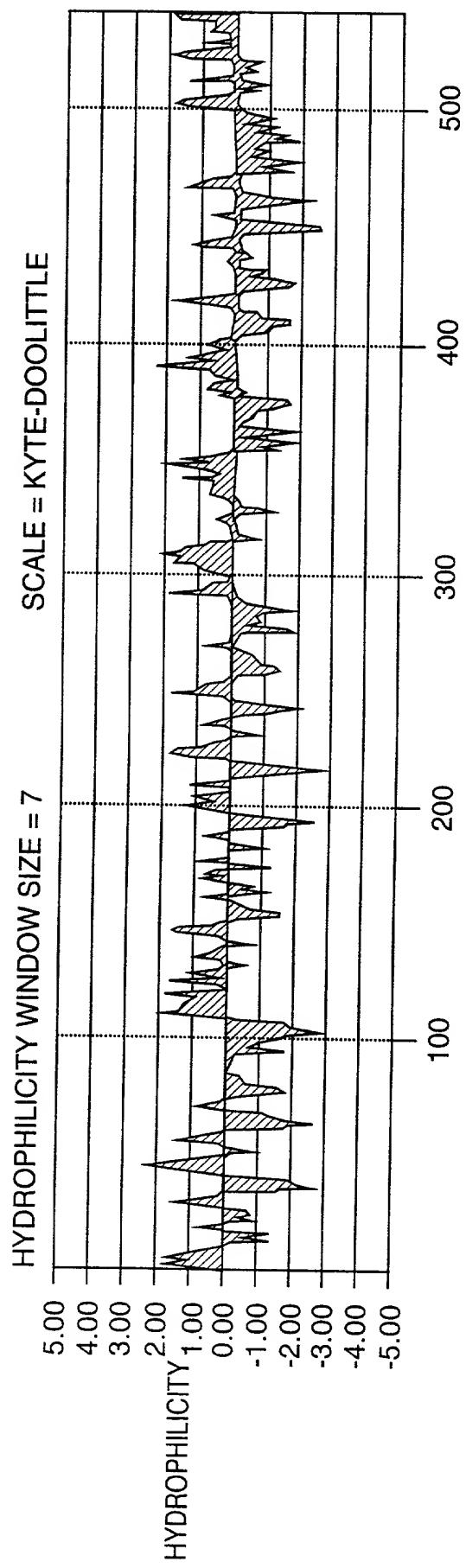


FIG. 3

Rabbit Tissue m RNPase

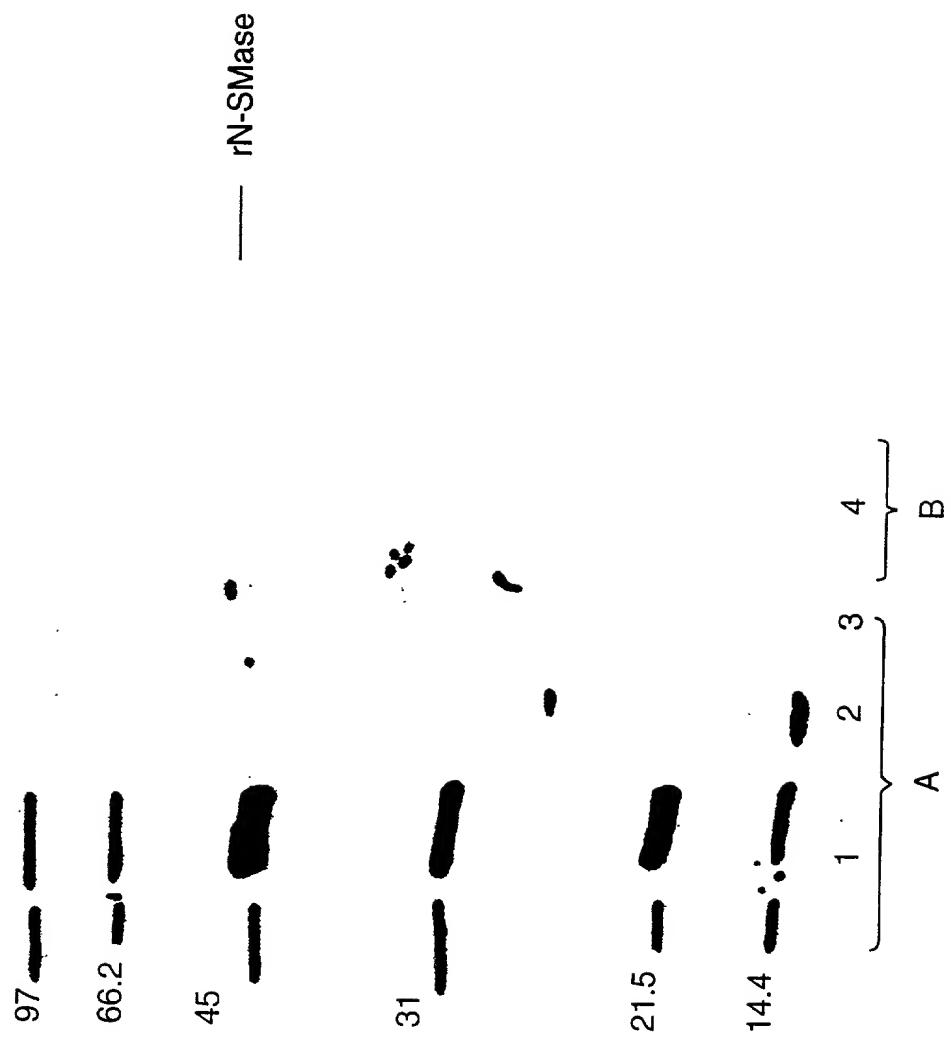


FIG. 4A

FIG. 4B

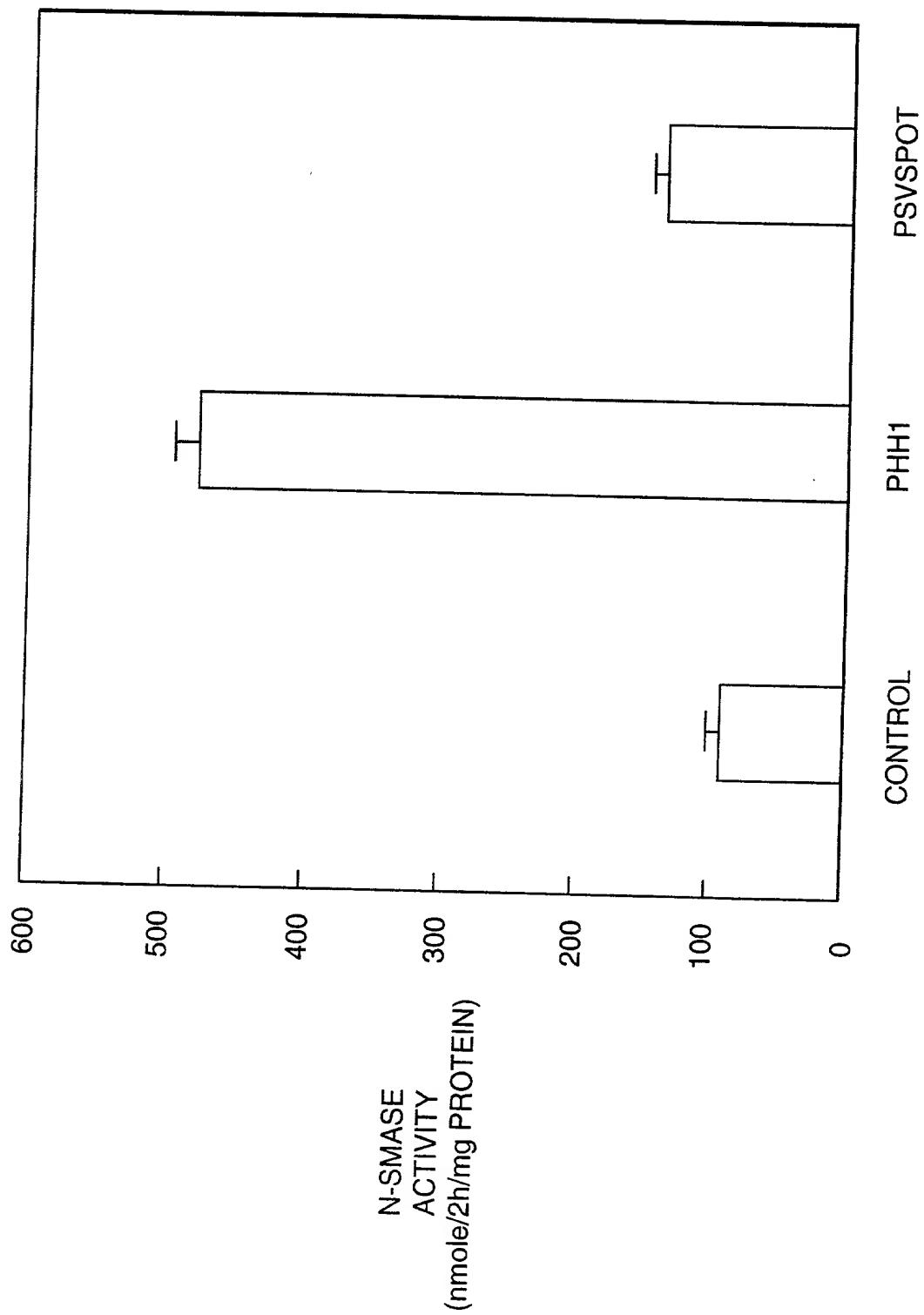


FIG. 5

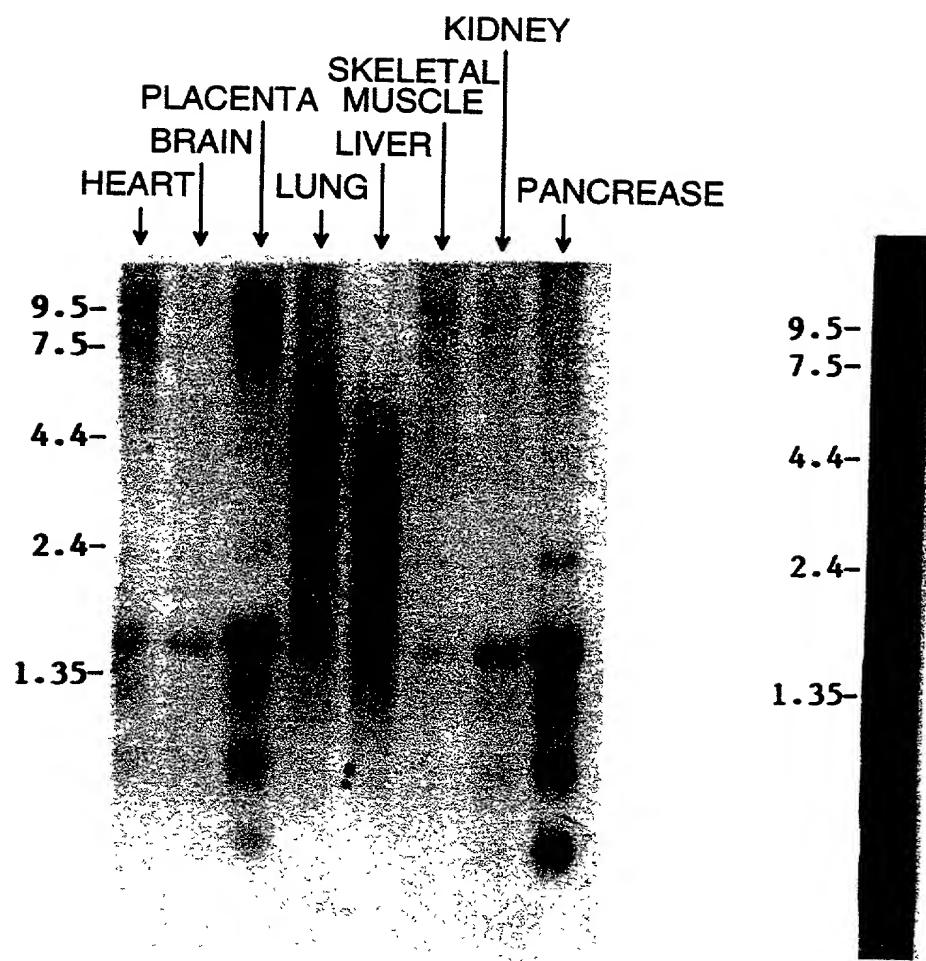


FIG. 6A

FIG. 6B

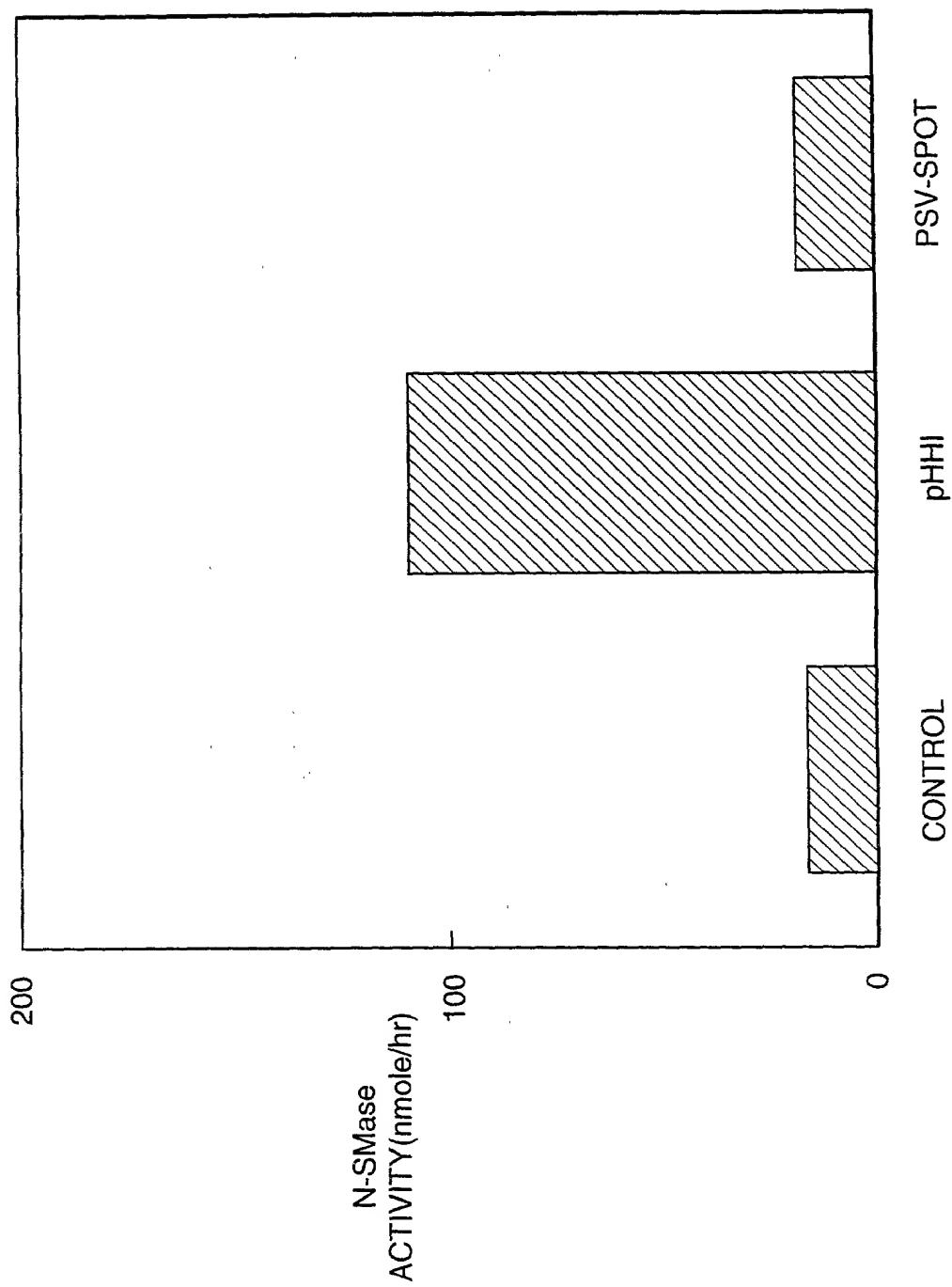


FIG. 7



FIG. 8A

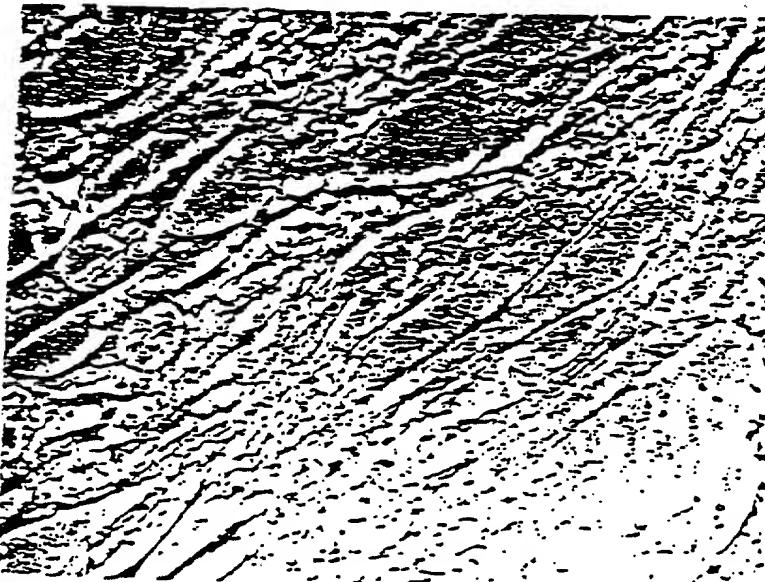


FIG. 8B

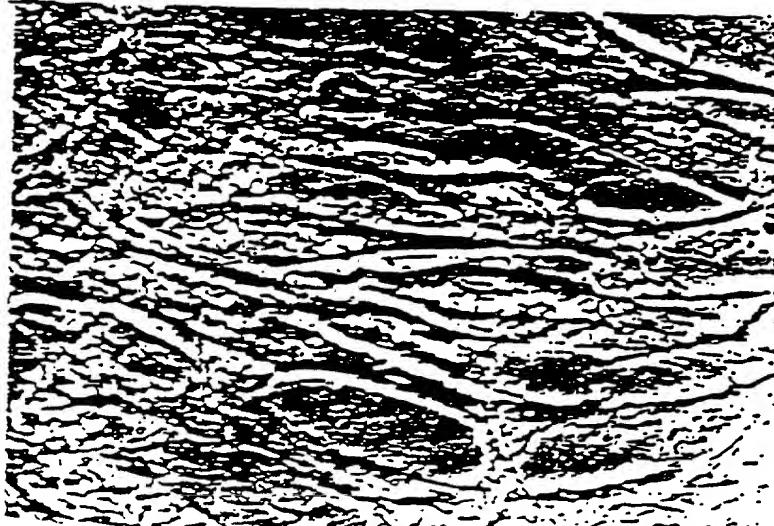


FIG. 8C

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-208 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME

which is described and claimed in:

the specification attached hereto.

the specification in U.S. Application Serial Number \_\_\_\_\_, filed on \_\_\_\_\_.

the specification in PCT international application Number \_\_\_\_\_,  
filed on \_\_\_\_\_; and was amended on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120				
U.S. Applications			Status (Check One)	
Application Serial No.	U.S. Filing Date		Patented	Pending
PCT Applications Designating the U.S.				
Application No.	Filing Date	U.S. Serial No. Assigned		

**CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)**  
(35 U.S.C. § 119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Peter F. Corless (Reg. No. 33,860)  
Sewall P. Bronstein (Reg. No. 16,919)  
David G. Conlin (Reg. No. 27,026)  
George W. Neuner (Reg. No. 26,964)

Ernest V. Linek (Reg. No. 29,822)  
Linda M. Buckley (Reg. No. 31,003)  
Ronald I. Eisenstein (Reg. No. 30,628)  
Henry D. Pahl, Jr. (Reg. No. 20,438)

Peter J. Manus (Reg. No. 26,766)  
David S. Resnick (Reg. No. 34,235)

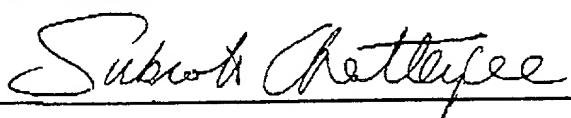
SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
Peter F. Corless Dike, Bronstein, Roberts & Cushman, LLP 130 Water Street Boston, Massachusetts 02109	Peter F. Corless (617) 523-3400

- 3 -

2 0 1	FULL NAME OF INVENTOR  Chatterjee	FIRST NAME  Subroto	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY  Columbia	STATE OR FOREIGN COUNTRY  Maryland	COUNTRY OF CITIZENSHIP  United States
POST OFFICE ADDRESS	POST OFFICE ADDRESS  6098 Sebring Drive	CITY  Columbia	STATE OR COUNTRY AND ZIP CODE  Maryland 20144

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201



Date:

12/23/96

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**In re application of: **S. Chatterjee**Serial No.: **0 8 /774,104** Group No.: **1814**Filed: **December 24, 1996** Examiner: **D. Mytelka**For: **RECOMBINANT N-SMASES AND NUCLEIC ACIDS ENCODING SAME****Box Sequence****Assistant Commissioner for Patents**  
**Washington, D.C. 20231****SUBMISSION OF "SEQUENCE LISTING," COMPUTER READABLE COPY,  
AND/OR AMENDMENT PERTAINING THERETO  
FOR BIOTECHNOLOGY INVENTION CONTAINING NUCLEOTIDE  
AND/OR AMINO ACID SEQUENCE**

(check and complete this item, if applicable)

1.  This replies to the Office Letter dated June 2, 1997.

NOTE: If these papers are filed before the office letter issues, adequate identification of the original papers should be made, e.g., in addition to the name of the inventor and title of invention, the filing date based on the "Express Mail" procedure, the serial number from the return post card or the attorney's docket number added.

 A copy of the Office Letter is enclosed.

---

**CERTIFICATION UNDER 37 CFR 1.8(a) and 1.10**

I hereby certify that, on the date shown below, this correspondence is being:

**MAILING** deposited with the United States Postal Service in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.**37 CFR 1.10** with sufficient postage as first class mail.  as "Express Mail Post Office to Addressee"  
Mailing Label No. \_\_\_\_\_**TRANSMISSION** transmitted by facsimile to the Patent and Trademark Office.Susan M. Dillon

Signature

Susan M. Dillon

(type or print name of person certifying)

Date: 7/2/97

**IDENTIFICATION OF DECLARANT**

2. I, Peter F. Corless  
(type or print name of declarant signing below)

state the following:

**ITEMS BEING SUBMITTED**

3. Submitted herewith is/are

(check each item as applicable)

- A.  "Sequence Listing(s)" for the nucleotide and/or amino acid sequence(s) in this application. Each "Sequence Listing" is assigned a separate identifier as required in 37 CFR § 1.821(c) and 37 CFR §§ 1.822 and 1.823.
- B.  An amendment to the description and/or claims, wherein reference is made to the sequence by use of the assigned identifier, as required in 37 CFR § 1.821(d).
- C.  A copy of each "Sequence Listing" submitted for this application in computer readable form, in accordance with the requirements of 37 CFR §§ 1.821(e) and 1.824.
- D.  Please transfer to this application, in accordance with 37 CFR § 1.821(e), the computer readable copy(ies) from applicant's other application identified as follows:

In re application of:

Serial No.: 0 /

Group No.:

Filed:

Examiner:

For:

The Computer readable form(s) of applicant's other application corresponds to the "Sequence Identifier(s)" of the application as follows:

Computer Readable Form  
(other application)

"Sequence Identifier"  
(this application)

(Submission—Nucleotide and/or Amino Acid Sequence [9-37]—page 2 of 6)

**NOTE:** "If the computer readable form of a new application is to be identical with the computer readable form of another application of the applicant on file in the Office, reference may be made to the other application and computer readable form in lieu of filing a duplicate computer readable form in the new application. The new application shall be accompanied by a letter making such reference to the other application and computer readable form, both of which shall be completely identified." 37 CFR 1.821(e).

E.  A statement that the content of each "Sequence Listing" submitted and each computer readable copy are the same, as required in 37 CFR § 1.821(g).  
 Because the statement is not made by a person registered to practice before the Office, the Statement is verified as required in 37 CFR § 1.821(b).

F.  Because this submission is made in fulfilling the requirement under 37 CFR § 1.821(g), a statement that the submission includes no new matter.  
 Because the statement is not made by a person registered to practice before the Office, the statement is verified, as required in 37 CFR § 1.821(g).

**STATEMENT THAT "SEQUENCE LISTING"  
AND COMPUTER READABLE COPY ARE THE SAME  
AND/OR THAT PAPERS SUBMITTED INCLUDES NO NEW MATTER**

4. I hereby state:

*(complete applicable item A and/or B)*

A.  Each computer readable form submitted in this application, including those forms requested to be transferred from applicant's other application, is the same as the "Sequence Listing" to which it is indicated to relate.

B.  All papers accompanying this submission, or for which a request for transfer from applicants' other application, introduce no new matter.

**VERIFICATION**

5. **NOTE:** "Such a statement must be verified statement if made by a person not registered to practice before the Office." 37 CFR § 1.821(f) and (g).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true: and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**STATUS**

6. Applicant is

a small entity. A verified statement:  
 is attached.  
 was already filed.  
 other than a small entity.

## EXTENSION OF TERM

7.

NOTE: "Extension of Time in Patent Cases (Supplement Amendments)—If a timely and complete response has been filed after a Non-Final Office Action, an extension of time is not required to permit filing and/or entry of an additional amendment after expiration of the shortened statutory period.

If a timely response has been filed after a Final Office Action, an extension of time is required to permit filing and/or entry of a Notice of Appeal or filing and/or entry of an additional amendment after expiration of the shortened statutory period unless the timely-filed response placed the application in condition for allowance. Of course, if a Notice of Appeal has been filed within the shortened statutory period, the period has ceased to run." Notice of December 10, 1985 (1061 O.G. 34-35).

NOTE: See 37 CFR 1.645 for extensions of time in interference proceedings and 37 CFR 1.550(c) for extensions of time in reexamination proceedings.

8. The proceedings herein are for a patent application and the provisions of 37 CFR 1.136 apply.

(complete (a) or (b) as applicable)

(a)  Applicant petitions for an extension of time under 37 CFR 1.136  
(fees: 37 CFR 1.17(a)-(d) for the total number of months checked below:

Extension <u>(months)</u>	Fee for other than <u>small entity</u>	Fee for <u>small entity</u>
<input type="checkbox"/> one month	\$ 110.00	\$ 55.00
<input type="checkbox"/> two months	\$ 380.00	\$190.00
<input type="checkbox"/> three months	\$ 900.00	\$450.00
<input type="checkbox"/> four months	\$ 1,400.00	\$700.00

Fee \$ \_\_\_\_\_

If an additional extension of time is required, please consider this a petition therefor.

(check and complete the next item, if applicable)

An extension for \_\_\_\_\_ months has already been secured and the fee paid therefor of \$\_\_\_\_\_ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request \$ \_\_\_\_\_

### OR

(b)  Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition for extension of time.

### FEE PAYMENT

9.  Attached is a check in the sum of \$\_\_\_\_\_

Charge Account No. \_\_\_\_\_ the sum of \$\_\_\_\_\_

A duplicate of this transmittal is attached.

## FEE DEFICIENCY

10.

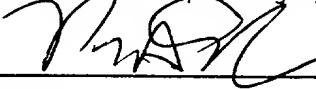
NOTE: If there is a fee deficiency and there is no authorization to charge an account, additional fees are necessary to cover the additional time consumed in making up the original deficiency. If the maximum, six-month period has expired before the deficiency is noted and corrected, the application is held abandoned. In those instances where authorization to charge is included, processing delays are encountered in returning the papers to the PTO Finance Branch in order to apply these charges prior to action on the cases. Authorization to charge the deposit account for any fee deficiency should be checked. See the Notice of April 7, 1986, 1065 O.G. 31-33.

11.  If any additional extension and/or fee is required, charge  
Account No. 04-1105

### SIGNATURE(s)

Peter F. Corless

(type or print name of person signing declaration)



Date

Signature

July 2, 1997

130 Water Street

P.O. Address of Signatory

Boston, MA 02109

(if applicable)

Telephone No. ( 617 ) 523-3400

Reg. No. 33,860

- Inventor(s)
- Assignee of complete interest
- Person authorized to sign on behalf of assignee
- Attorney or agent of record
- Filed under Rule 34(a)
- Other \_\_\_\_\_

(specify identity of declarant)

(complete the following, if applicable)

\_\_\_\_\_  
(type name of assignee)

\_\_\_\_\_  
Address of assignee

\_\_\_\_\_  
Title of person authorized to sign on behalf of assignee

A "CERTIFICATE UNDER 37 CFR 3.73(b)" is attached.

Assignment recorded in PTO on \_\_\_\_\_

Reel \_\_\_\_\_ Frame \_\_\_\_\_



---

**SIGNATURE OF ATTORNEY**

Reg. No.: 33,860

Peter F. Corless

(type or print name of attorney)

Tel. No.: (617 ) 523-3400

130 Water Street

**P.O. Address**

Boston, MA 02109

## SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Chatterjee, Subroto

(ii) TITLE OF THE INVENTION: RECOMBINANT N-SMASES AND NUCLEIC ACIDS  
ENCODING SAME

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
- (B) STREET: 130 Water Street
- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/774,104  
(B) FILING DATE: 12/24/96  
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Corless, Peter F  
(B) REGISTRATION NUMBER: 33,860  
(C) REFERENCE/DOCKET NUMBER: 46906

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-523-3400  
(B) TELEFAX: 617-523-6440  
(C) TELEX:

(2) INFORMATION FOR SEO ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1197 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

SEQUENCE LISTING

- (1) GENERAL INFORMATION

(i) APPLICANT: Chatterjee, Subroto

(ii) TITLE OF THE INVENTION: RECOMBINANT N-SMASES AND NUCLEIC ACIDS  
ENCODING SAME

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
- (B) STREET: 130 Water Street
- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Corless, Peter F
- (B) REGISTRATION NUMBER: 33,860
- (C) REFERENCE/DOCKET NUMBER: 46906

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 617-523-3400
- (B) TELEFAX: 617-523-6440
- (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1197 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY:

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGATGACAT	ATCACGAAAC	GCGCGCGTTG	GCTCAAAGCG	ACTTACAGCA	ACTCTATGCG	60
GCACTTGAAA	CAACTGAATT	TGGCGCTTAC	TTTGCACAC	CCGCTGATGA	TACTTTACGT	120
TTTGGCATTG	GCGCAATCGC	TACGGCAAAA	ACGGCTCAGG	CATTACAAGG	TGCGGTTGTT	180
TTTGGTGCAG	AGTCATTGAG	TGAACAAGAG	TACCCGCAGT	CTGAATTGAT	GGCGGGTTTT	240
TGGTTTGTCC	CCGAAGTGAT	GGTGACCATC	GCGGCAGATA	AAATCACGTT	CGGATCAGAT	300
ACCGTATCTG	ATTTTACGAC	GTGGCTGGCG	CAGTTCGTGC	AAAACAGCC	AAATACGGTG	360
ACCACTAGTC	ATGTGACAGA	TGAAGTGGAT	TGGATCGAAC	GGACAGAGAA	TTTGATTGAT	420
ACCTTAGCCA	TCGATCAAAC	CTTAGCCAAA	GTCGTTTTG	GTCGGCAACA	GACCTGCA	480
TTATCCGACA	CGTTACGACT	GGCACAAATT	ATTCTGCGT	TAGCTGAGCA	GGCGAATACG	540
TATCATGTGG	TTTTAAAGCG	ACATGATGAA	TTGTTTATT	CAGCAACACC	GGAACGGTTA	600
GTGGCTATGT	CAGGTGGTCA	GATCGCTACG	GCGGCGGTG	CTGGGACAAG	CCGGCGCGGG	660
ACGGATGGCG	CTGACGATAT	CGCGTTAGGC	GAAGCGTTGT	TAGCCAGTCA	GAAAAACCGC	720
ATTGAACATC	AATATGTCGT	GGCAAGTATC	ACGACACGCT	TGCAAGACGT	GACGACGTCG	780
CTAAAGGTGC	CGGCCATGCC	AAGTTTACTC	AAAAATAAGC	AAGTTCAGCA	TTTGTACACA	840
CCAATTACAG	GGGACATTGC	GGCACATTTA	AGTGTGACCG	CGATTGTTGA	CCGCTTGCAT	900
CCAACACCA	CACTGGGTGG	CGTCCCACGT	GAAGCGGCC	TGTATTACAT	TGCGACCCAT	960
GAGAAGACAC	CTCGTGGCTT	GTTCAGGT	CCTATTGGCT	ATTTACCGC	AGATAATAGT	1020
GGGAATTG	TGGTTGGCAT	CCGTTCCATG	TATGTGAATC	AAACGCAGCG	ACGAGCAACT	1080
TTATTTGCTG	GTGCCGGGAT	TGTGGCTGAC	TCCGATGCGC	AACAAGAATA	TGAAGAAACT	1140
GGGTTGAAAT	TTGAACCCAT	GCGGCAATTG	TTAAAGGACT	ACAATCATGT	CGAATGA	1197

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 397 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY:

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Met	Thr	Tyr	His	Glu	Thr	Arg	Ala	Leu	Ala	Gln	Ser	Asp	Leu	Gln
1					5				10						15
Gln	Leu	Tyr	Ala	Ala	Leu	Glu	Thr	Thr	Glu	Phe	Gly	Ala	Tyr	Phe	Ala
									25						30
Thr	Pro	Ala	Asp	Asp	Thr	Leu	Arg	Phe	Gly	Ile	Gly	Ala	Ile	Ala	Thr
								35	40						45
Ala	Lys	Thr	Ala	Gln	Ala	Leu	Gln	Gly	Ala	Val	Phe	Gly	Ala	Gln	Ser
								50	55						60

Phe Asp Glu Gln Glu Tyr Pro Gln Ser Glu Leu Met Ala Gly Phe Trp  
 65 70 75 80  
 Phe Val Pro Glu Val Met Val Thr Ile Ala Ala Asp Lys Ile Thr Phe  
 85 90 95  
 Gly Ser Asp Thr Val Ser Asp Phe Thr Thr Trp Leu Ala Gln Phe Val  
 100 105 110  
 Pro Lys Gln Pro Asn Thr Val Thr Thr Ser His Val Thr Asp Glu Val  
 115 120 125  
 Asp Trp Ile Glu Arg Thr Glu Asn Leu Ile Asp Thr Leu Ala Ile Asp  
 130 135 140  
 Gln Thr Leu Ala Lys Val Val Phe Gly Arg Gln Gln Thr Leu Gln Leu  
 145 150 155 160  
 Ser Asp Thr Leu Arg Leu Ala Gln Ile Ile Arg Ala Leu Ala Glu Gln  
 165 170 175  
 Ala Asn Thr Tyr His Val Val Leu Lys Arg His Asp Glu Leu Phe Ile  
 180 185 190  
 Ser Ala Thr Pro Glu Arg Leu Val Ala Met Ser Gly Gly Gln Ile Ala  
 195 200 205  
 Thr Ala Ala Val Ala Gly Thr Ser Arg Arg Gly Thr Asp Gly Ala Asp  
 210 215 220  
 Asp Ile Ala Leu Gly Glu Ala Leu Leu Ala Ser Gln Lys Asn Arg Ile  
 225 230 235 240  
 Glu His Gln Tyr Val Val Ala Ser Ile Thr Thr Arg Leu Gln Asp Val  
 245 250 255  
 Thr Thr Ser Leu Lys Val Pro Ala Met Pro Ser Leu Leu Lys Asn Lys  
 260 265 270  
 Gln Val Gln His Leu Tyr Thr Pro Ile Thr Gly Asp Ile Ala Ala His  
 275 280 285  
 Leu Ser Val Thr Ala Ile Val Asp Arg Leu His Pro Thr Pro Ala Leu  
 290 295 300  
 Gly Gly Val Pro Arg Glu Ala Ala Leu Tyr Tyr Ile Ala Thr His Glu  
 305 310 315 320  
 Lys Thr Pro Arg Gly Leu Phe Ala Gly Pro Ile Gly Tyr Phe Thr Ala  
 325 330 335  
 Asp Asn Ser Gly Glu Phe Val Val Gly Ile Arg Ser Met Tyr Val Asn  
 340 345 350  
 Gln Thr Gln Arg Arg Ala Thr Leu Phe Ala Gly Ala Gly Ile Val Ala  
 355 360 365  
 Asp Ser Asp Ala Gln Gln Glu Tyr Glu Glu Thr Gly Leu Lys Phe Glu  
 370 375 380  
 Pro Met Arg Gln Leu Leu Lys Asp Tyr Asn His Val Glu  
 385 390 395

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY:

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Leu Lys Val Pro Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 7 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY:

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Ile Thr Val Arg Val  
1 5

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY:

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGCGGCACT ATTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAATGCC AAAACGTA

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCATGAT GACATATCAC GAAACGCGCG TTTCGTGATA TGTCAATCATG

50

- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGATGACAT	ATCACGAAAC	GCGCGCGTTG	GCTCAAAGCG	ACTTACAGCA	ACTCTATGCG	60
GCACTTGAAA	CAACTGAATT	TGGCGCTTAC	TTTGCACAC	CCGCTGATGA	TACTTACGT	120
TTTGGCATTG	GCGCAATCGC	TACGGCAAAA	ACGGCTCAGG	CATTACAAGG	TGCGGTTGTT	180
TTTGGTGCAC	AGTCATTGA	TGAACAAGAG	TACCCGCAGT	CTGAATTGAT	GGCGGGTTTT	240
TGGTTTGTCC	CCGAAGTGT	GGTGACCATC	GCAGCAGATA	AAATCACGTT	CGGATCAGAT	300
ACCGTATCTG	ATTTTACGAC	GTGGCTGGCG	CAGTCGTGC	AAAACAGCC	AAATAACGGTG	360
ACCACTAGTC	ATGTGACAGA	TGAAGTGGAT	TGGATCGAAC	GGACAGAGAA	TTTGATTGAT	420
ACCTTAGCCA	TCGATCAAAC	CTTAGCCAAA	GTCGTTTTG	GTCGGCAACA	GACCCTGCAG	480
TTATCCGACA	CGTTACGACT	GGCACAAATT	ATTCGTGCGT	TAGCTGAGCA	GGCGAATACG	540
TATCATGTGG	TTTTAAAGCG	ACATGATGAA	TTGTTTATT	CAGCAACACC	GGAACGGTTA	600
GTGGCTATGT	CAGGTGGTCA	GATCGCTACG	GCGCGGGTCG	CTGGGACAAG	CCGGCGCGGG	660
ACGGATGGCG	CTGACGATAT	CGCGTTAGGC	GAAGCGTTGT	TAGCCAGTCA	GAAAAACCGC	720
ATTGAACATC	AATATGTCGT	GGCAAGTATC	ACGACACGCT	TGCAAGACGT	GACGACGTG	780
CTAAAGGTGC	CGGCCATGCC	AAGTTTACTC	AAAAATAAGC	AAGTCAGCA	TTTGTACACA	840
CCAATTACAG	GGGACATTGC	GGCACATT	AGTGTGACCG	CGATTGTTGA	CCGCTTGCAT	900
CCAACACCA	CACTGGGTGG	CGTCCCACGT	GAAGCGGCC	TGTATTACAT	TGCGACCCAT	960
GAGAAGACAC	CTCGTGGCTT	GTGTCAGGT	CCTATTGGCT	ATTTTACCGC	AGATAATAGT	1020
GGGAAATTG	TGGTTGGCAT	CCGTTCCATG	TATGTGAATC	AAACGCAGCG	ACGAGCAACT	1080
TTATTTGCTG	GTGCCGGGAT	TGTGGCTGAC	TCCGATGCGC	AACAAGAATA	TGAAGAAACT	1140
GGGTTGAAAT	TTGAACCCAT	GCGGCAATTG	TTAAAGGACT	ACAATCATGT	CGAATGA	1197

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 397 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Met	Thr	Tyr	His	Glu	Thr	Arg	Ala	Leu	Ala	Gln	Ser	Asp	Leu	Gln	
1					5				10					15		
Gln	Leu	Tyr	Ala	Ala	Leu	Glu	Thr	Thr	Glu	Phe	Gly	Ala	Tyr	Phe	Ala	
														20	25	30
Thr	Pro	Ala	Asp	Asp	Thr	Leu	Arg	Phe	Gly	Ile	Gly	Ala	Ile	Ala	Thr	
														35	40	45
Ala	Lys	Thr	Ala	Gln	Ala	Leu	Gln	Gly	Ala	Val	Phe	Gly	Ala	Gln	Ser	
														50	55	60
Phe	Asp	Glu	Gln	Glu	Tyr	Pro	Gln	Ser	Glu	Leu	Met	Ala	Gly	Phe	Trp	

	65	70	75	80											
Phe	Val	Pro	Glu	Val	Met	Val	Thr	Ile	Ala	Ala	Asp	Lys	Ile	Thr	Phe
					85				90					95	
Gly	Ser	Asp	Thr	Val	Ser	Asp	Phe	Thr	Thr	Trp	Leu	Ala	Gln	Phe	Val
					100			105					110		
Pro	Lys	Gln	Pro	Asn	Thr	Val	Thr	Thr	Ser	His	Val	Thr	Asp	Glu	Val
					115			120					125		
Asp	Trp	Ile	Glu	Arg	Thr	Glu	Asn	Leu	Ile	Asp	Thr	Leu	Ala	Ile	Asp
					130			135			140				
Gln	Thr	Leu	Ala	Lys	Val	Val	Phe	Gly	Arg	Gln	Gln	Thr	Leu	Gln	Leu
					145			150			155			160	
Ser	Asp	Thr	Leu	Arg	Leu	Ala	Gln	Ile	Ile	Arg	Ala	Leu	Ala	Glu	Gln
					165			170					175		
Ala	Asn	Thr	Tyr	His	Val	Val	Leu	Lys	Arg	His	Asp	Glu	Leu	Phe	Ile
					180			185					190		
Ser	Ala	Thr	Pro	Glu	Arg	Leu	Val	Ala	Met	Ser	Gly	Gly	Gln	Ile	Ala
					195			200				205			
Thr	Ala	Ala	Val	Ala	Gly	Thr	Ser	Arg	Arg	Gly	Thr	Asp	Gly	Ala	Asp
					210			215			220				
Asp	Ile	Ala	Leu	Gly	Glu	Ala	Leu	Leu	Ala	Ser	Gln	Lys	Asn	Arg	Ile
					225			230			235			240	
Glu	His	Gln	Tyr	Val	Val	Ala	Ser	Ile	Thr	Thr	Arg	Leu	Gln	Asp	Val
					245			250					255		
Thr	Thr	Ser	Leu	Lys	Val	Pro	Ala	Met	Pro	Ser	Leu	Leu	Lys	Asn	Lys
					260			265				270			
Gln	Val	Gln	His	Leu	Tyr	Thr	Pro	Ile	Thr	Gly	Asp	Ile	Ala	Ala	His
					275			280			285				
Leu	Ser	Val	Thr	Ala	Ile	Val	Asp	Arg	Leu	His	Pro	Thr	Pro	Ala	Leu
					290			295			300				
Gly	Gly	Val	Pro	Arg	Glu	Ala	Ala	Leu	Tyr	Tyr	Ile	Ala	Thr	His	Glu
					305			310			315			320	
Lys	Thr	Pro	Arg	Gly	Leu	Phe	Ala	Gly	Pro	Ile	Gly	Tyr	Phe	Thr	Ala
					325			330					335		
Asp	Asn	Ser	Gly	Glu	Phe	Val	Val	Gly	Ile	Arg	Ser	Met	Tyr	Val	Asn
					340			345					350		
Gln	Thr	Gln	Arg	Arg	Ala	Thr	Leu	Phe	Ala	Gly	Ala	Gly	Ile	Val	Ala
					355			360			365				
Asp	Ser	Asp	Ala	Gln	Gln	Glu	Tyr	Glu	Glu	Thr	Gly	Leu	Lys	Phe	Glu
					370			375			380				
Pro	Met	Arg	Gln	Leu	Leu	Lys	Asp	Tyr	Asn	His	Val	Glu			
					385			390			395				

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Leu Lys Val Pro Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Ile Thr Val Arg Val  
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGCGGGCACT ATTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAATGCC AAAACGTA

18

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCATGAT GACATATCAC GAAACGCGCG TTTCGTGATA TGTCATCATG

50

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: Chatterjee, Subroto

(ii) TITLE OF THE INVENTION: RECOMBINANT N-SMASES AND NUCLEIC ACIDS  
ENCODING SAME

(iii) NUMBER OF SEQUENCES: 7

## (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
- (B) STREET: 130 Water Street
- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/774,104
- (B) FILING DATE: 12/24/96
- (C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Corless, Peter F
- (B) REGISTRATION NUMBER: 33,860
- (C) REFERENCE/DOCKET NUMBER: 46906

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 617-523-3400
- (B) TELEFAX: 617-523-6440
- (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1197 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

## (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGATGACAT	ATCACGAAAC	GCGCGCGTTG	GCTCAAAGCG	ACTTACAGCA	ACTCTATGCG	60
GCACTTGAAA	CAACTGAATT	TGGCGCTTAC	TTTGCACAC	CCGCTGATGA	TACTTTACGT	120
TTTGGCATTG	GCGCAATCGC	TACGGCAAAA	ACGGCTCAGG	CATTACAAGG	TGCGGTTGTT	180
TTTGGTGCAG	AGTCATTGAG	TGAACAAAGAG	TACCCGAGT	CTGAATTGAT	GGCGGGTTTT	240
TGGTTTGTCC	CCGAAGTGAT	GGTGACCATC	GCGGCAGATA	AAATCACGTT	CGGATCAGAT	300
ACCGTATCTG	ATTTTACGAC	GTGGCTGGCG	CAGTCGTGC	AAAACAGCC	AAATACGGTG	360
ACCACTAGTC	ATGTGACAGA	TGAAGTGGAT	TGGATCGAAC	GGACAGAGAA	TTTGATTGAT	420
ACCTTAGCCA	TCGATCAAAC	CTTAGCCAAA	GTCGTTTTG	GTCGGCAACA	GACCCCTGCAG	480
TTATCCGACA	CGTTACGACT	GGCACAAATT	ATTCTGTCGT	TAGCTGAGCA	GGCGAATACG	540
TATCATGTGG	TTTTAAAGCG	ACATGATGAA	TTGTTTATT	CAGCAACACC	GGAACGGTTA	600
GTGGCTATGT	CAGGTGGTCA	GATCGCTACG	GCGGCAGGTG	CTGGGACAAG	CCGGCGCGGG	660
ACGGATGGCG	CTGACGATAT	CGCGTTAGGC	GAAGCGTTGT	TAGCCAGTCA	GAAAAACCGC	720
ATTGAACATC	AATATGTCGT	GGCAAGTATC	ACGACACGCT	TGCAAGACGT	GACGACGTG	780
CTAAAGGTGC	CGGCCATGCC	AAGTTTACTC	AAAAATAAGC	AAGTTCAAGCA	TTTGTACACA	840
CCAATTACAG	GGGACATTG	GGCACATT	AGTGTGACCG	CGATTGTTGA	CCGCTTGCAT	900
CCAACACCA	CACTGGTGG	CGTCCCACGT	GAAGCGGCC	TGTATTACAT	TGCGACCCAT	960
GAGAAGACAC	CTCGTGGCTT	GTTCGCAGGT	CCTATTGGCT	ATTTTACCGC	AGATAATAGT	1020
GGGGAATTG	TGGTTGGCAT	CCGTTCCATG	TATGTGAATC	AAACGCAGCG	ACGAGCAACT	1080
TTATTTGCTG	GTGCCGGGAT	TGTGGCTGAC	TCCGATGCGC	AACAAGAATA	TGAAGAAACT	1140
GGGTTGAAAT	TTGAACCCAT	GCGCAATTG	TTAAAGGACT	ACAATCATGT	CGAATGA	1197

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 397 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Met	Thr	Tyr	His	Glu	Thr	Arg	Ala	Leu	Ala	Gln	Ser	Asp	Leu	Gln
1					5						10				15
Gln	Leu	Tyr	Ala	Ala	Leu	Glu	Thr	Thr	Glu	Phe	Gly	Ala	Tyr	Phe	Ala

20	25	30	
Thr Pro Ala Asp Asp Thr Leu Arg Phe Gly Ile Gly Ala Ile Ala Thr			
35	40	45	
Ala Lys Thr Ala Gln Ala Leu Gln Gly Ala Val Phe Gly Ala Gln Ser			
50	55	60	
Phe Asp Glu Gln Glu Tyr Pro Gln Ser Glu Leu Met Ala Gly Phe Trp			
65	70	75	80
Phe Val Pro Glu Val Met Val Thr Ile Ala Ala Asp Lys Ile Thr Phe			
85	90	95	
Gly Ser Asp Thr Val Ser Asp Phe Thr Thr Trp Leu Ala Gln Phe Val			
100	105	110	
Pro Lys Gln Pro Asn Thr Val Thr Thr Ser His Val Thr Asp Glu Val			
115	120	125	
Asp Trp Ile Glu Arg Thr Glu Asn Leu Ile Asp Thr Leu Ala Ile Asp			
130	135	140	
Gln Thr Leu Ala Lys Val Val Phe Gly Arg Gln Gln Thr Leu Gln Leu			
145	150	155	160
Ser Asp Thr Leu Arg Leu Ala Gln Ile Ile Arg Ala Leu Ala Glu Gln			
165	170	175	
Ala Asn Thr Tyr His Val Val Leu Lys Arg His Asp Glu Leu Phe Ile			
180	185	190	
Ser Ala Thr Pro Glu Arg Leu Val Ala Met Ser Gly Gly Gln Ile Ala			
195	200	205	
Thr Ala Ala Val Ala Gly Thr Ser Arg Arg Gly Thr Asp Gly Ala Asp			
210	215	220	
Asp Ile Ala Leu Gly Glu Ala Leu Leu Ala Ser Gln Lys Asn Arg Ile			
225	230	235	240
Glu His Gln Tyr Val Val Ala Ser Ile Thr Thr Arg Leu Gln Asp Val			
245	250	255	
Thr Thr Ser Leu Lys Val Pro Ala Met Pro Ser Leu Leu Lys Asn Lys			
260	265	270	
Gln Val Gln His Leu Tyr Thr Pro Ile Thr Gly Asp Ile Ala Ala His			
275	280	285	
Leu Ser Val Thr Ala Ile Val Asp Arg Leu His Pro Thr Pro Ala Leu			
290	295	300	
Gly Gly Val Pro Arg Glu Ala Ala Leu Tyr Tyr Ile Ala Thr His Glu			
305	310	315	320
Lys Thr Pro Arg Gly Leu Phe Ala Gly Pro Ile Gly Tyr Phe Thr Ala			
325	330	335	
Asp Asn Ser Gly Glu Phe Val Val Gly Ile Arg Ser Met Tyr Val Asn			
340	345	350	
Gln Thr Gln Arg Arg Ala Thr Leu Phe Ala Gly Ala Gly Ile Val Ala			
355	360	365	
Asp Ser Asp Ala Gln Gln Glu Tyr Glu Glu Thr Gly Leu Lys Phe Glu			
370	375	380	
Pro Met Arg Gln Leu Leu Lys Asp Tyr Asn His Val Glu			
385	390	395	

(2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Leu Lys Val Pro Ala

1 5

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Ile Thr Val Arg Val

1 5

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGCGGGCACT ATTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAATGCC AAAACGTA

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCATGAT GACATATCAC GAAACGCGCG TTTCGTGATA TGTCATCATG

50